METHODS OF TREATING CONDITIONS ASSOCIATED WITH AN EDG-1 RECEPTOR

[0001] This application is entitled to and claims the benefit of U.S. Provisional Application No. 60/397,299, filed July, 18, 2002, which is hereby incorporated by reference in its entirety.

1. FIELD OF INVENTION

[0002] The present invention relates generally to methods of modulating biological activity mediated by the Edg-1 receptor. More specifically, the present invention provides compounds and compositions, which may be used to selectively modulate, *e.g.*, agonize or antagonize, the Edg-1 receptor. The present invention also provides methods for making these compounds.

2. BACKGROUND OF THE INVENTION

[0003] Recent studies have revealed a complex biological role for cell membrane phospholipids, which were previously believed to have only a structural function. Following cell activation, membrane phospholipids may be metabolized to eicosanoids and lysophospholipids, which are important regulators of cellular function and behavior. Lysophospholipids include compounds such as lysophosphatidic acid ("LPA"), sphingosine-1-phosphate ("S1P"), lysophosphatidylcholine and sphingosylphosphorylcholine and are important second messengers that can activate particular cell surface transmembrane G-protein coupled receptors known as endothelial gene differentiation ("Edg") receptors.

[0004] Two distinct subfamilies of G-protein coupled receptors bind LPA or S1P specifically and transduce diverse cellular signals by associating with one or more G proteins. Based on amino acid sequence identities, S1P1 (Edg-1), S1P3 (Edg 3), S1P2 (Edg 5), and S1P5 (Edg 8) belong to one structural cluster and LPA1 (Edg 2), LPA2 (Edg 4) and LPA3 (Edg 7) are members of a second structural cluster (Goetzl, E. J., and Lynch, K. R. **2000**, *Ann. N. Y. Acad. Sci.* 905:1-357). Members of both subfamilies range in size from about 351 to about 400 amino acids, and are encoded by chromosomes 1, 9 or 19. The amino acid sequence of S1P4 (Edg 6) lies between those of the two major clusters by amino acid sequence identity (Graler *et al.*, **1998**, *Genomics* 53:164-169). Currently, there are three known Edg receptors specifically activated by LPA (LPA1 or Edg 2, LPA2 or Edg 4 and LPA3 or Edg 7)

and five known S1P receptors specifically activated by S1P (S1P1 or Edg-1, S1P2 or Edg 5, S1P3 or Edg 3, S1P4 or Edg 6, and S1P5 or Edg 8).

[0005] Edg-1 (human Edg-1, GenBank Accession No. AF233365), Edg-3 (human Edg-3, GenBank Accession No. X83864), Edg-5 (human Edg-5, GenBank Accession No. AF034780), Edg-6 (human Edg-6, GenBank Accession No. AJ000479) and Edg-8 (human Edg-8, GenBank Accession No. AF317676) receptors are activated by S1P, while LPA activates Edg-2 (human Edg-2, GenBank Accession No., U78192), Edg-4 (human Edg-4, GenBank Accession Nos. AF233092 or AF011466) and Edg-7 (human Edg-7, GenBank Accession No. AF127138) receptors.

[0006] Edg receptors are believed to mediate critical cellular events such as cell proliferation and cell migration, which makes these receptors attractive therapeutic targets. However, currently known compounds, which bind to Edg receptors, are almost exclusively phospholipids (*e.g.*, S1P and LPA, analogs of S1P and LPA, dioctyl glycerol, *etc*). Most of these phospholipids compounds fail to effectively discriminate between different Edg receptors and have poor physicochemical properties, which limits their potential use as pharmaceutical agents. Thus, there exists a need for compounds, which are not phospholipids that bind or otherwise regulate Edg receptors and can also selectively bind to a specific Edg receptor.

3. SUMMARY OF THE INVENTION

[0007] The present invention provides compounds that modulate the S1P1 or Edg-1 receptor (*e.g.*, human Edg-1, GenBank Accession No. AF233365). Such compounds preferably selectively bind or otherwise modulate the Edg-1 receptor.

[0008] In one aspect, the present invention provides methods for modulating Edg-1 receptor mediated biological activity. The present invention also provides methods for using Edg-1 modulators (*i.e.*, agonists or antagonists) in treating or preventing diseases such as ovarian cancer, peritoneal cancer, endometrial cancer, cervical cancer, breast cancer, colorectal cancer, uterine cancer, stomach cancer, small intestine cancer, thyroid cancer, lung cancer, kidney cancer, pancreas cancer and prostrate cancer; acute lung diseases, adult respiratory distress syndrome ("ARDS"), acute inflammatory exacerbation of chronic lung diseases such as asthma, surface epithelial cell injury, (*e.g.*, transcorneal freezing or cutaneous burns) and

cardiovascular diseases (*e.g.*, ischemia) in a subject in need of such treatment or prevention.

[0009] In another aspect, the present invention provides methods for using Edg-1 modulators (*i.e.*, agonists or antagonists) in treating or preventing disorders such as, but not limited to, vasoconstriction in cerebral arteries, autoimmune and related immune disorders, including, but not limited to, systemic lupus erythematosus, rheumatoid arthritis, non-glomerular nephrosis, psoriasis, chronic active hepatitis, ulcerative colitis, Crohn's disease, Behçet's disease, chronic glomerulonephritis, chronic thrombocytopenic purpura, and autoimmune hemolytic anemia. Additionally, Edg-1 antagonists can also be used in organ transplantation. In yet another embodiment, Edg-1 agonists and antagonists can be used to treat vascular occlusive disorders. For example, activation of Edg-1 receptors by using an Edg-1 agonist can result in increased vasoconstriction which is beneficial in conditions such as migraine headaches. Inhibition of Edg-1 by an Edg-1 antagonist can be beneficial in conditions such as a stroke, a subarachnoid hemorrhage, or a vasospasm such as a cerebral vasospasm.

[0010] In still another aspect, the present invention provides methods for using Edg-1 modulators (*i.e.*, agonists or antagonists) to treat or prevent a disease or disorder in a subject, comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of an Edg-1 modulator, *e.g.*, for example, an Edg-1 agonist, that stimulates the immune system. In certain embodiments, the subject is afflicted by an infectious agent. In other embodiments, the subject is immunocompromised.

[0011] In yet another aspect, the present invention provides methods for using Edg-1 modulators (*i.e.*, agonists or antagonists) to treat or prevent an immune disorder in a subject, comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of an Edg-1 modulator, *e.g.*, for example, an Edg-1 antagonist, wherein the immune disorder is characterized by inappropriate activation of the immune system. The immune disorder can be any immune disorder characterized by inappropriate activation of the immune system known by one of skill in the art without limitation. In certain embodiments, the subject is the recipient of a transplanted cell, tissue, or organ.

[0012] In still another aspect, the present invention provides a method of modulating an Edg-1 receptor mediated biological activity in a cell. A cell expressing

the Edg-1 receptor is contacted with an amount of an Edg-1 receptor modulator sufficient to modulate the Edg-1 receptor mediated biological activity.

[0013] In yet another aspect, the present invention provides a method for modulating an Edg-1 receptor mediated biological activity in a subject. In such a method, an amount of a modulator of the Edg-1 receptor effective to modulate an Edg-1 receptor mediated biological activity is administered to the subject.

4. BRIEF DESCRIPTION OF THE FIGURES

[0014] **Figure 1** illustrates the selectivity of **101** for the Edg-1 receptor; and

[0015] **Figure 2** illustrates the selectivity of **102** for the Edg-1 receptor; and

[0016] **Figure 3** illustrates the selectivity of **103** for the Edg-1 receptor; and

[0017] **Figure 4** provides the effect of Edg-1 antagonist **101** on calcium response in human umbilical vein endothelial cells; and

[0018] **Figure 5** provides the effect of Edg-1 antagonist **102** on calcium response in human umbilical vein endothelial cells; and

[0019] **Figure 6** provides the effect of 10 μM Edg-1 antagonist **102** on S1P-stimulated matrigel invasion of human umbilical vein endothelial cells; and

[0020] **Figure 7** provides the effect of Edg-1 agonist **104** on the invasion of human umbilical vein endothelial cells; and

[0021] **Figure 8** provides the effect of Edg-1 antagonist **101** on Edg-1 agonist **104** stimulated invasion by human umbilical vein endothelial cells; and

[0022] **Figure 9** provides the effect of Edg-1 agonist **104** on migration of human peripheral blood mononuclear cells ("PMBCs"); and

[0023] **Figure 10** provides the effects of Edg-1 antagonist **101**, Edg-3 antagonist **301**, and the combination of **101** and **301** on S1P stimulated invasion by human vein endothelial cells; and

[0024] **Figure 11** provides the effects of Edg-1 agonist **104** on murine CD4 T cell chemotactic responses to exodus-2; and

[0025] **Figure 12** provides the effects of FTY720 and the Edg-1 antagonist **102** on S1P regulation of murine CD4 T cell chemotactic responses to exodus-2; and

[0026] **Figure 13** illustrates the migration of PBMCs to S1P; and

[0027] **Figure 14** illustrates the regulation by Edg-1 of lymphocyte migration *in vitro*; and

[0028] **Figure 15** illustrates the regulation by Edg-1 of lymphocyte migration in mouse dorsal air-pouches.

5. BRIEF DESCRIPTION OF THE TABLES

[0029] **Table 1** provides compounds **105-113**;

[0030] **Table 2** provides selectivity of compounds **101, 102** and **103** for Edg-1;

[0031] **Table 3** provides binding assays; and

[0032] **Table 4** provides selectivity of compound **104** for Edg-1.

6. DETAILED DESCRIPTION OF THE INVENTION

6.1. Definitions

[0033] "Compounds of the invention" refers generally to any modulator of the S1P1 or Edg-1 receptor (e.g., human Edg-1, GenBank Accession No. AF233365) and includes any Edg-1 receptor modulator encompassed by generic formulae disclosed herein and further includes any species within those formulae whose structure is disclosed herein. The compounds of the invention may be identified either by their chemical structure and/or chemical name. If the chemical structure and chemical name conflict, the chemical structure is determinative of the identity of the compound. The compounds of the invention may contain one or more chiral centers and/or double bonds and, therefore, may exist as stereoisomers, such as double-bond isomers (*i.e.*, geometric isomers), enantiomers or diastereomers. Accordingly, the chemical structures depicted herein encompass all possible enantiomers and stereoisomers of the illustrated compounds, including the stereoisomerically pure form (*e.g.*, geometrically pure, enantiomerically pure or diastereomerically pure) and enantiomeric and stereoisomeric mixtures. Enantiomeric and stereoisomeric mixtures can be resolved into their component enantiomers or stereoisomers using separation techniques or chiral synthesis techniques well known to the skilled artisan. The compounds of the invention may also exist in several tautomeric forms including, but not limited to, the enol form, the keto form and mixtures thereof. Accordingly, the compounds of the invention as described herein encompass all possible tautomeric forms of the illustrated chemical structures. The compounds of the invention also include isotopically labeled compounds where one or more atoms have an atomic mass different from the atomic mass conventionally found in nature. Examples of isotopes that may be incorporated in the compounds of the invention include, but are

not limited to, ^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{15}N, ^{18}O, ^{17}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F and ^{36}Cl. Further, it should be understood that when partial structures of the compounds of the invention are illustrated, brackets indicate the point of attachment of the partial structure to the rest of the compound.

[0034] "Composition of the invention" refers to at least one compound of the invention and a pharmaceutically acceptable vehicle, with which the compound is administered to a subject. When administered to a subject, the compounds of the invention are administered in isolated form, which means that the compounds are separated from a synthetic organic reaction mixture.

[0035] "Alkyl" refers to a saturated or unsaturated, branched, straight-chain or cyclic monovalent hydrocarbon group derived by the removal of one hydrogen atom from a single carbon atom of a parent alkane, alkene or alkyne. Typical alkyl groups include, but are not limited to, methyl; ethyls such as ethanyl, ethenyl, ethynyl; propyls such as propan-1-yl, propan-2-yl, cyclopropan-1-yl, prop-1-en-1-yl, prop-1-en-2-yl, prop-2-en-1-yl (allyl), cycloprop-1-en-1-yl; cycloprop-2-en-1-yl, prop-1-yn-1-yl , prop-2-yn-1-yl, *etc.*; butyls such as butan-1-yl, butan-2-yl, 2-methyl-propan-1-yl, 2-methyl-propan-2-yl, cyclobutan-1-yl, but-1-en-1-yl, but-1-en-2-yl, 2-methyl-prop-1-en-1-yl, but-2-en-1-yl , but-2-en-2-yl, buta-1,3-dien-1-yl, buta-1,3-dien-2-yl, cyclobut-1-en-1-yl, cyclobut-1-en-3-yl, cyclobuta-1,3-dien-1-yl, but-1-yn-1-yl, but-1-yn-3-yl, but-3-yn-1-yl, *etc.*; and the like.

[0036] The term "alkyl" is specifically intended to include groups having any degree or level of saturation, *i.e.*, groups having exclusively single carbon-carbon bonds, groups having one or more double carbon-carbon bonds, groups having one or more triple carbon-carbon bonds and groups having mixtures of single, double and triple carbon-carbon bonds. Where a specific level of saturation is intended, the expressions "alkanyl," "alkenyl," and "alkynyl" are used. Preferably, an alkyl group comprises from 1 to 20 carbon atoms.

[0037] "Alkanyl" refers to a saturated branched, straight-chain or cyclic alkyl group derived by the removal of one hydrogen atom from a single carbon atom of a parent alkane. Typical alkanyl groups include, but are not limited to, methanyl; ethanyl; propanyls such as propan-1-yl, propan-2-yl (isopropyl), cyclopropan-1-yl, *etc.*; butanyls such as butan-1-yl, butan-2-yl (*sec*-butyl), 2-methyl-propan-1-yl (isobutyl), 2-methyl-propan-2-yl (*t*-butyl), cyclobutan-1-yl, *etc.*; and the like.

[0038] "Alkenyl" refers to an unsaturated branched, straight-chain or cyclic alkyl group having at least one carbon-carbon double bond derived by the removal of one hydrogen atom from a single carbon atom of a parent alkene. The group may be in either the *cis* or *trans* conformation about the double bond(s). Typical alkenyl groups include, but are not limited to, ethenyl; propenyls such as prop-1-en-1-yl, prop-1-en-2-yl, prop-2-en-1-yl (allyl), prop-2-en-2-yl, cycloprop-1-en-1-yl; cycloprop-2-en-1-yl; butenyls such as but-1-en-1-yl, but-1-en-2-yl, 2-methyl-prop-1-en-1-yl, but-2-en-1-yl , but-2-en-1-yl, but-2-en-2-yl, buta-1,3-dien-1-yl, buta-1,3-dien-2-yl, cyclobut-1-en-1-yl, cyclobut-1-en-3-yl, cyclobuta-1,3-dien-1-yl, *etc.*; and the like.

[0039] "Alkynyl" refers to an unsaturated branched, straight-chain or cyclic alkyl group having at least one carbon-carbon triple bond derived by the removal of one hydrogen atom from a single carbon atom of a parent alkyne. Typical alkynyl groups include, but are not limited to, ethynyl; propynyls such as prop-1-yn-1-yl, prop-2-yn-1-yl, *etc.*; butynyls such as but-1-yn-1-yl, but-1-yn-3-yl, but-3-yn-1-yl , *etc.*; and the like.

[0040] "Acyl" refers to a radical -C(O)R, where R is hydrogen, alkyl, cycloalkyl, cycloheteroalkyl, aryl, arylalkyl, heteroalkyl, heteroaryl, heteroarylalkyl as defined herein. Representative examples include, but are not limited to, formyl, acetyl, cylcohexylcarbonyl, cyclohexylmethylcarbonyl, benzoyl, benzylcarbonyl and the like.

[0041] "Acylamino" refers to a radical -NR'C(O)R, where R' is hydrogen, alkyl, cycloalkyl, cycloheteroalkyl, aryl, arylalkyl, heteroalkyl, heteroaryl, heteroarylalkyl and R is hydrogen, alkyl, alkoxy, cycloalkyl, cycloheteroalkyl, aryl, arylalkyl, heteroalkyl, heteroaryl or heteroarylalkyl, as defined herein. Representative examples include, but are not limited to, formylamino, acetylamino, cylcohexylcarbonylamino, cyclohexylmethyl-carbonylamino, benzoylamino, benzylcarbonylamino and the like.

[0042] "Alkylamino" refers to a radical -NHR where R represents an alkyl or cycloalkyl group as defined herein. Representative examples include, but are not limited to, methylamino, ethylamino, 1-methylethylamino, cyclohexyl amino and the like.

[0043] "Alkoxy" refers to a radical -OR where R represents an alkyl or cycloalkyl group as defined herein. Representative examples include, but are not limited to, methoxy, ethoxy, propoxy, butoxy, cyclohexyloxy and the like.

[0044] "Alkoxyamino" refers to a radical –N(H)OR where R represents an alkyl or cycloalkyl group as defined herein.

[0045] "Alkoxycarbonyl" refers to a radical -C(O)-alkoxy where alkoxy is as defined herein.

[0046] "Alkylarylamino" refers to a radical -NRR' where R represents an alkyl or cycloalkyl group and R' is an aryl as defined herein.

[0047] "Alkylsulfonyl" refers to a radical $-S(O)_2R$ where R is an alkyl or cycloalkyl group as defined herein. Representative examples include, but are not limited to, methylsulfonyl, ethylsulfonyl, propylsulfonyl, butylsulfonyl and the like.

[0048] "Alkylsulfinyl" refers to a radical -S(O)R where R is an alkyl or cycloalkyl group as defined herein. Representative examples include, but are not limited to, methylsulfinyl, ethylsulfinyl, propylsulfinyl, butylsulfinyl and the like.

[0049] "Alkylthio" refers to a radical -SR where R is an alkyl or cycloalkyl group as defined herein that may be optionally substituted as defined herein. Representative examples include, but are not limited to, methylthio, ethylthio, propylthio, butylthio, and the like.

[0050] "Amino" refers to the radical $-NH_2$.

[0051] "Aryl" refers to a monovalent aromatic hydrocarbon group derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Typical aryl groups include, but are not limited to, groups derived from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene, chrysene, coronene, fluoranthene, fluorene, hexacene, hexaphene, hexalene, *as*-indacene, *s*-indacene, indane, indene, naphthalene, octacene, octaphene, octalene, ovalene, penta-2,4-diene, pentacene, pentalene, pentaphene, perylene, phenalene, phenanthrene, picene, pleiadene, pyrene, pyranthrene, rubicene, triphenylene, trinaphthalene and the like. Preferably, an aryl group comprises from 6 to 20 carbon atoms.

[0052] "Arylalkyl" refers to an acyclic alkyl group in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp^3 carbon atom, is replaced with an aryl group. Typical arylalkyl groups include, but are not limited to, benzyl, 2-phenylethan-1-yl, 2-phenylethen-1-yl, naphthylmethyl,

2-naphthylethan-1-yl, 2-naphthylethen-1-yl, naphthobenzyl, 2-naphthophenylethan-1-yl and the like. Where specific alkyl moieties are intended, the nomenclature arylalkanyl, arylalkenyl and/or arylalkynyl is used. Preferably, an arylalkyl group is (C_6-C_{30}) arylalkyl, *e.g.*, the alkanyl, alkenyl or alkynyl moiety of the arylalkyl group is (C_1-C_{10}) and the aryl moiety is (C_6-C_{20}).

[0053] "Arylalkyloxy" refers to an -O-arylalkyl radical where arylalkyl is as defined herein.

[0054] "Arylamino" means a radical -NHR where R represents an aryl group as defined herein.

[0055] "Aryloxycarbonyl" refers to a radical -C(O)-O-aryl where aryl is as defined herein.

[0056] "Arylsulfonyl" refers to a radical -$S(O)_2$R where R is an aryl or heteroaryl group as defined herein.

[0057] "Azido" refers to the radical -N3.

[0058] "Carbamoyl" refers to the radical -C(O)N(R)2 where each R group is independently hydrogen, alkyl, cycloalkyl or aryl, as defined herein, which may be optionally substituted as defined herein.

[0059] "Carboxy" refers to the radical -C(O)OH.

[0060] "Carboxyamino" refers to the radical –N(H)C(O)OH.

[0061] "Cyanato" refers to the radical -OCN.

[0062] "Cyano" refers to the radical -CN.

[0063] "Cycloalkyl" refers to a saturated or unsaturated cyclic alkyl group. Where a specific level of saturation is intended, the nomenclature "cycloalkanyl" or "cycloalkenyl" is used. Typical cycloalkyl groups include, but are not limited to, groups derived from cyclopropane, cyclobutane, cyclopentane, cyclohexane, and the like. In a preferred embodiment, the cycloalkyl group is (C_3-C_{10}) cycloalkyl, more preferably (C_3-C_6) cycloalkyl.

[0064] "Cycloheteroalkyl" refers to a saturated or unsaturated cyclic alkyl group in which one or more carbon atoms (and any associated hydrogen atoms) are independently replaced with the same or different heteroatom. Typical heteroatoms to replace the carbon atom(s) include, but are not limited to, N, P, O, S, Si, *etc.* Where a specific level of saturation is intended, the nomenclature "cycloheteroalkanyl" or "cycloheteroalkenyl" is used. Typical cycloheteroalkyl groups include, but are not limited to, groups derived from dioxanes, dioxolanes, epoxides, imidazolidine,

morpholine, piperazine, piperidine, pyrazolidine, pyrrolidine, quinuclidine, tetrahydrofuran, tetrahydropyran and the like.

[0065] "Cycloheteroalkyloxycarbonyl" refers to a radical -C(O)-OR where R is cycloheteroalkyl is as defined herein.

[0066] "Dialkylamino" means a radical -NRR' where R and R' independently represent an alkyl, substituted alkyl, aryl, substituted aryl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, heteroaryl, or substituted heteroaryl group as defined herein.

[0067] "Halo" means fluoro, chloro, bromo, or iodo.

[0068] "Haloalkyl" means an alkyl radical substituted by one or more halo atoms wherein alkyl and halo is as defined herein.

[0069] "Heteroalkyloxy" means an -O-heteroalkyl group where heteroalkyl is as defined herein.

[0070] "Heteroalkyl, Heteroalkanyl, Heteroalkenyl, Heteroalkynyl" refer to alkyl, alkanyl, alkenyl and alkynyl groups, respectively, in which one or more of the carbon atoms (and any associated hydrogen atoms) are each independently replaced with the same or different heteroatomic groups. Typical heteroatomic groups include, but are not limited to, -O-, -S-, -O-O-, -S-S-, -O-S-, -NR'-, =N-N=, -N=N-, -N=N-NR'-, -PH-, $-P(O)_2-$, $-O-P(O)_2-$, -S(O)-, $-S(O)_2-$, $-SnH_2-$ and the like, wherein R' is hydrogen, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, aryl or substituted aryl.

[0071] "Heteroaryl" refers to a monovalent heteroaromatic group derived by the removal of one hydrogen atom from a single atom of a parent heteroaromatic ring system. Typical heteroaryl groups include, but are not limited to, groups derived from acridine, arsindole, carbazole, ∃-carboline, chromane, chromene, cinnoline, furan, imidazole, indazole, indole, indoline, indolizine, isobenzofuran, isochromene, isoindole, isoindoline, isoquinoline, isothiazole, isoxazole, naphthyridine, oxadiazole, oxazole, perimidine, phenanthridine, phenanthroline, phenazine, phthalazine, pteridine, purine, pyran, pyrazine, pyrazole, pyridazine, pyridine, pyrimidine, pyrrole, pyrrolizine, quinazoline, quinoline, quinolizine, quinoxaline, tetrazole, thiadiazole, thiazole, thiophene, triazole, xanthene, and the like. Preferably, the heteroaryl group is between 5-20 membered heteroaryl, with 5-10 membered heteroaryl being particularly preferred. Preferred heteroaryl groups are those derived from thiophene,

pyrrole, benzothiophene, benzofuran, indole, pyridine, quinoline, imidazole, oxazole and pyrazine.

[0072] "Heteroaryloxy" refers to an -O-heteroarylalkyl radical where heteroarylalkyl is as defined herein.

[0073] "Heteroaryloxycarbonyl" refers to a radical -C(O)-OR where R is heteroaryl as defined herein.

[0074] "Heteroarylalkyl" refers to an acyclic alkyl group in which one of the hydrogen atoms bonded to a carbon atom, typically a terminal or sp^3 carbon atom, is replaced with a heteroaryl group. Where specific alkyl moieties are intended, the nomenclature heteroarylalkanyl, heteroarylalkenyl and/or heterorylalkynyl is used. In preferred embodiments, the heteroarylalkyl group is a 6-30 membered heteroarylalkyl, *e.g.*, the alkanyl, alkenyl or alkynyl moiety of the heteroarylalkyl is 1-10 membered and the heteroaryl moiety is a 5-20 membered heteroaryl.

[0075] "Hydroxy" refers to the radical -OH.

[0076] "Leaving group" has the meaning conventionally associated with it in synthetic organic chemistry, i.e., an atom or a group capable of being displaced by a nucleophile and includes halo (such as chloro, bromo, and iodo), alkoxycarbonyl (*e.g.*, acetoxy), aryloxycarbonyl, mesyloxy, tosyloxy, trifluoromethanesulfonyloxy, aryloxy (*e.g.*, 2,4-dinitrophenoxy), methoxy, N,O-dimethylhydroxylamino, and the like.

[0077] "Nitro" refers to the radical –NO2.

[0078] "Oxo" refers to the divalent radical =O.

[0079] "Pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans.

[0080] "Pharmaceutically acceptable salt" refers to a salt of a compound of the invention that is pharmaceutically acceptable and that possesses the desired pharmacological activity of the parent compound. Such salts include: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl)

benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethane-disulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic acid, glucoheptonic acid, 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; or (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, *e.g.*, an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, N-methylglucamine and the like.

[0081] "Pharmaceutically acceptable vehicle" refers to a diluent, adjuvant, excipient or carrier with which a compound of the invention is administered.

[0082] "Preventing" or "prevention" refers to a reduction in risk of acquiring a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop in a subject that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease).

[0083] "Prodrug" refers to a pharmacologically inactive derivative of a drug molecule that requires a transformation within the body to release the active drug. Typically, prodrugs are designed to overcome pharmaceutical and/or pharmacokinetically based problems associated with the parent drug molecule that would otherwise limit the clinical usefulness of the drug.

[0084] "Promoiety" refers to a form of protecting group that when used to mask a functional group within a drug molecule converts the drug into a prodrug. Typically, the promoiety will be attached to the drug *via* bond(s) that are cleaved by enzymatic or non-enzymatic means *in vivo*. Ideally, the promoiety is rapidly cleared from the body upon cleavage from the prodrug.

[0085] "Protecting group" refers to a grouping of atoms that when attached to a reactive group in a molecule masks, reduces or prevents that reactivity. Examples of protecting groups can be found in Green *et al.*, "Protective Groups in Organic Chemistry", (Wiley, 2nd ed. 1991) and Harrison *et al.*, "Compendium of Synthetic Organic Methods", Vols. 1-8 (John Wiley and Sons, 1971-1996). Representative amino protecting groups include, but are not limited to, formyl, acetyl, trifluoroacetyl, benzyl, benzyloxycarbonyl ("CBZ"), *tert*-butoxycarbonyl ("Boc"), trimethylsilyl

("TMS"), 2-trimethylsilyl-ethanesulfonyl ("SES"), trityl and substituted trityl groups, allyloxycarbonyl, 9-fluorenylmethyloxycarbonyl ("FMOC"), nitro-veratryloxycarbonyl ("NVOC") and the like. Representative hydroxy protecting groups include, but are not limited to, those where the hydroxy group is either acylated or alkylated such as benzyl, and trityl ethers as well as alkyl ethers, tetrahydropyranyl ethers, trialkylsilyl ethers and allyl ethers.

[0086] "Subject" includes humans. The terms "human," "patient" and "subject" are used interchangeably herein.

[0087] "Substituted" refers to a group in which one or more hydrogen atoms are each independently replaced with the same or different substituent(s). Typical substituents include, but are not limited to, -X, -R_{14}, -O^-, =O, -OR_{14}, -SR_{14}, -S^-, =S, -$NR_{14}R_{15}$, =NR_{14}, -CX_3, -CF_3, -CN, -OCN, -SCN, -NO, -NO_2, =N_2, -N_3, -$S(O)_2O^-$, -$S(O)_2OH$, -$S(O)_2R_{14}$, -$OS(O_2)O^-$, -$OS(O)_2R_{14}$, -$P(O)(O^-)_2$, -$P(O)(OR_{14})(O^-)$, -$OP(O)(OR_{14})(OR_{15})$, -$C(O)R_{14}$, -$C(S)R_{14}$, -$C(O)OR_{14}$, -$C(O)NR_{14}R_{15}$, -$C(O)O^-$, -$C(S)OR_{14}$, -$NR_{16}C(O)NR_{14}R_{15}$, -$NR_{16}C(S)NR_{14}R_{15}$, -$NR_{17}C(NR_{16})NR_{14}R_{15}$ and -$C(NR_{16})NR_{14}R_{15}$, where each X is independently a halogen; each R_{14}, R_{15}, R_{16} and R_{17} are independently hydrogen, alkyl, substituted alkyl, aryl, substituted alkyl, arylalkyl, substituted alkyl, cycloalkyl, substituted alkyl, cycloheteroalkyl, substituted cycloheteroalkyl, heteroalkyl, substituted heteroalkyl, heteroaryl, substituted heteroaryl, heteroarylalkyl, substituted heteroarylalkyl, -$NR_{18}R_{19}$, -$C(O)R_{18}$ or -$S(O)_2R_{18}$ or optionally R_{18} and R_{19} together with the atom to which they are both attached form a cycloheteroalkyl or substituted cycloheteroalkyl ring; and R_{18} and R_{19} are independently hydrogen, alkyl, substituted alkyl, aryl, substituted alkyl, arylalkyl, substituted alkyl, cycloalkyl, substituted alkyl, cycloheteroalkyl, substituted cycloheteroalkyl, heteroalkyl, substituted heteroalkyl, heteroaryl, substituted heteroaryl, heteroarylalkyl or substituted heteroarylalkyl.

[0088] "Sulfonyl" refers to the divalent radical -S(O2)-.

[0089] "Therapeutically effective amount" means the amount of a compound that, when administered to a subject for treating a disease, is sufficient to effect such treatment for the disease. The "therapeutically effective amount" can vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

[0090] "Thio" refers to the radical -SH.

[0091] "Thiocyanato" refers to the radical -SCN.

[0092] "Thiono" refers to the divalent radical =S.

[0093] "Treating" or "treatment" of any disease or disorder refers, in one embodiment, to ameliorating the disease or disorder (*i.e.*, arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment "treating" or "treatment" refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, "treating" or "treatment" refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both. In yet another embodiment, "treating" or "treatment" refers to delaying the onset of the disease or disorder.

[0094] Reference will now be made in detail to preferred embodiments of the invention. While the invention will be described in conjunction with the preferred embodiments, it will be understood that it is not intended to limit the invention to those preferred embodiments. To the contrary, it is intended to cover alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

6.2. The Use of the Compounds of the Invention

[0095] The present invention provides a method of modulating an S1P1 or Edg-1 receptor (*e.g.*, human Edg-1, GenBank Accession No. AF233365) mediated biological activity. A cell expressing the Edg-1 receptor is contacted with an amount of an Edg-1 receptor agonist or antagonist sufficient to modulate an Edg-1 receptor mediated biological activity.

[0096] Those of skill in the art will appreciate that Edg-1 is a G protein coupled receptor ("GPCR"). The Edg-1 (S1P1) receptor is encoded by an endothelial differentiation gene and along with related receptors, Edg-3 (S1P3), Edg-5 (S1P2), Edg-6 (S1P4) and Edg-8 (S1P5), binds sphingosine-1-phosphate ("S1P"). Preferably, the Edg-1 receptor is a human receptor.

[0097] The Edg-1 receptor may be expressed by recombinant DNA methods well known to those of skill in the art. Particularly useful cell types for expressing and assaying Edg-1 include, but are not limited to, HTC4 (rat hepatoma cells), RH7777 (rat hepatoma cells), HepG2 (human hepatoma cells), CHO (Chinese hamster ovary cells) and HEK-293 (human embryonic kidney cells). Particularly useful vectors for expressing G-protein receptors include, but are not limited to,

pLXSN and pCMV (Clontech Labs, Palo Alto, CA; Invitrogen Corporation, Carlsbad, CA).

[0098] DNA encoding Edg-1 is well known (*e.g.*, human Edg-1, GenBank Accession No. AF233365) and can be transfected into human or mammalian cells according to methods known to those of skill in the art. For example, DNA encoding human Edg-1 can be co-transfected with a standard packaging vector, such as those described above, which provides an ecotropic envelope for viral replication, into a packaging cell line such as GP-293 (Clontech Labs, Palo Alto, CA).

[0099] Alternatively, DNA encoding Edg-1 can be transfected into the EcoPack-293 cell line which has, in addition to *gag* and *pol*, the *env* gene to produce an ecotropic envelope. Both methods (*i.e.*, co-transfection with a packaging vector or use of EcoPack-293) enable the production of an ecotropic envelope for viral packaging, and can thus advantageously be used to transfect rat and mouse cells. For use in human and other mammalian cells, AmphoPack-293 cell line can be used (Clontech, Palo Alto, CA).

[0100] A number of natural cell lines naturally express Edg-1 receptors. These include, but are not limited to, CaOV-3 human ovarian cancer cells, MDA-MB-453 and MDA-MB-231 breast cancer cells, HT-1080 human fibrosarcoma, HUVEC cells, SKOV3 human ovarian cancer cells, A2780 human ovarian cells, Hela human cervical adenocarcinoma cells, HEK293 human embryonic kidney cells, NIH 3T3 mouse fibroblast cells (ATCC, Manassas, VA; Vec Technologies Inc., Rensselaer, NY; Dr. Edward Goetzl, University of California, San Francisco, San Francisco, CA).

[0101] Those of skill in the art will appreciate that cells which express the Edg-1 receptor may be grown *in vitro* or may be part of a complex organism such as, for example, a mammal. It is contemplated that the methods of the current invention will be applicable to inhibition of Edg-1 receptor activity, regardless of the local environment. In one preferred embodiment, cells that express the Edg-1 receptor are grown *in vitro* (*i.e.*, are cultured). In another preferred embodiment, cells that express the Edg-1 receptor are *in vivo* (*i.e.*, are part of a complex organism).

[0102] The cells in which the method of the invention may be practiced include, but are not limited to, hepatoma cells, ovarian cells, epithelial cells, fibroblast cells, neuronal cells, carcinoma cells, pheochromocytoma cells, myoblast cells, platelet cells, keratinocytes and fibrosarcoma cells. More specifically, the cells in which the invention may be practiced include, but are not limited to, SKOV3 human

ovarian cells, HTC rat hepatoma cells, CAOV-3 human ovarian cancer cells, A2780 human ovarian cells, MDA-MB-453 breast cancer cells, MDA-MB-231 breast cancer cells, HUVEC, Hela human cervical adenocarcinoma cells, HEK293 human embryonic kidney cells, NIH 3T3 mouse fibroblast cells and HT-1080 human fibrosarcoma cells. Additional cells for the practice of the method of the invention include those that are described in co-pending U.S. application no. 09/904,099, filed July 11, 2001, the content of which is hereby incorporated by reference in its entirety.

[0103] In a second aspect of the invention, an Edg-1 receptor mediated biological activity is modulated in a subject or in an animal model. A therapeutically effective amount of a modulator of the Edg-1 receptor is administered to the subject or animal. Preferably, the subject or animal is in need of such treatment.

[0104] The biological activity mediated by the Edg-1 receptor may include, for example, calcium mobilization, VEGF synthesis, IL-8 synthesis, platelet activation, cell migration, phosphoinositide hydrolysis, inhibition of cAMP formation or actin polymerization. Preferably, the biological activity mediated by the Edg-1 receptor also includes, but is not limited to, apoptosis, angiogenesis, wound healing, inflammation, expression of endogenous protein growth factors, cancer invasiveness or atherogenesis. Most preferably, the biological activity mediated by the Edg-1 receptor is cell proliferation, which may lead to enhancement of wound healing; alternatively, it may lead ovarian cancer, peritoneal cancer, endometrial cancer, cervical cancer, breast cancer, colorectal cancer, uterine cancer, stomach cancer, small intestine cancer, thyroid cancer, lung cancer, kidney cancer, pancreas cancer or prostrate cancer. In one embodiment, cell proliferation is stimulated by S1P.

[0105] In another embodiment, the biological activity mediated by the Edg-1 receptor may include increasing fatty acids levels (*e.g.*, free fatty acids and lyso-phosphatidylcholine) which may lead to acute lung diseases, such as adult respiratory distress syndrome ("ARDS") and acute inflammatory exacerbation of chronic lung diseases like asthma.

[0106] In yet other embodiments, the biological activity mediated by the Edg-1 receptor can be an immune response. In certain embodiments, the immune response can be stimulated by Edg-1 receptor modulators. The Edg-1 receptor modulators that can stimulate an immune response are generally Edg-1 receptor agonists; however, certain Edg-1 receptor antagonists may also be able to stimulate an immune response.

[0107] One of skill in the art can readily recognize subjects that will benefit from stimulation of an immune response. For example, subjects that suffer from an inherited immune deficiency will benefit from stimulation of an immune response. Other such subjects include subjects infected with a virus. For example, the subject can be infected with cytomegalovirus, herpes simplex virus I, herpes simplex virus II, influenza A virus, influenza B virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, or human immunodeficiency virus. In yet other embodiments, the subject that will benefit from stimulation of an immune response include subjects that are administered a vaccine. In such embodiments, an Edg-1 receptor modulator can be administered as an adjuvant to a vaccine. In other embodiments, an Edg-1 receptor modulator can be administered simultaneously with a vaccine.

[0108] In other embodiments, the immune response can be suppressed by Edg-1 receptor modulators. In such embodiments, the Edg-1 receptor modulators can be either Edg-1 receptor agonists or antagonists. Without intending to be bound by any particular theory or mechanism of action, it is believed that sufficiently strong Edg-1 agonists or sufficiently high doses of Edg-1 agonists can suppress an immune response by desensitizing cells that express the Edg-1 receptor to signaling by S1P. Edg-1 antagonists are thought to suppress an immune response by inhibiting S1P binding to the Edg-1 receptor or otherwise preventing the Edg-1 receptor from transducing a signal.

[0109] One of skill in the art can readily recognize subjects that will benefit from suppression of an immune response. For example, such subjects include those that suffer from an immune disorder that is characterized by an inappropriate activation of the immune system. Such disorders include, but are not limited to, systemic lupus erythematosus, rheumatic cardiatis, polymyosis, pemphigus, bullous dermatits herpetiformis, Stevens-Johnson syndrome, mycosis fungoides, dermatitis, ulcerative colitis, Crohn's disease, intractable sprue, idiopathic thrombocytopenic purpura, hemolytic anemia, erythroblastopenia, congenital hypoplastic anemia, osteoarthritis, rheumatoid arthritis, bursitis, acute gouty arthritis, epicondylitis, acute nonspecific tenosynovitis, multiple sclerosis, keratitis, irititis, irisocyclitis, chorioretinitis, choroiditis, optic neuritis, sarcoiodosis, Loeffler's syndrome, berylliosis, tuberculosis, sponylitis, tenosynovitis, psoriatic arthritis, and type I diabetes mellitus. Another example of a subject that will benefit from suppression of

an immune response is a subject that is the recipient of a transplanted cell, tissue, or organ.

[0110] In yet another embodiment, the present invention provides methods for using Edg-1 modulators in treating or preventing disorders such as, but not limited to, vasoconstriction in cerebral arteries, autoimmune and related immune disorders, including, but not limited to, systemic lupus erythematosus, rheumatoid arthritis, non-glomerular nephrosis, psoriasis, chronic active hepatitis, ulcerative colitis, Crohn's disease, Behçet's disease, chronic glomerulonephritis, chronic thrombocytopenic purpura, and autoimmune hemolytic anemia. Additionally, Edg-1 modulators can also be used in organ transplantation. In yet another embodiment, Edg-1 agonists and antagonists can be used to treat vascular occlusive disorders. For example, activation of Edg-1 receptors by using an Edg-1 agonist will result in increased vasoconstriction which is beneficial in conditions such as migraine headaches. Inhibition of Edg-1 by an Edg-1 antagonist will be beneficial in conditions such as a stroke, a subarachnoid hemorrhage, or a vasospasm such as a cerebral vasospasm.

[0111] In one embodiment, the modulator exhibits inhibitory selectivity for the Edg-1 receptor. For example, the modulator exhibits at least about 5 fold inhibitory selectivity for Edg-1 relative to other Edg receptors. Inhibitory selectivity can be measured by assays such as a calcium mobilization assay or a migration and/or invasion assay or a proliferation assay, for example, as described in Sections 7.10, 7.12 and 7.13, respectively. Other assays suitable for determining inhibitory selectivity would be known to one of skill in the art. Preferred assays include the calcium mobilization assay of Section 7.10.

[0112] In another embodiment, the modulator exhibits at least about 20 fold inhibitory selectivity for Edg-1 relative to other Edg receptors.

[0113] In another embodiment, the modulator exhibits at least about 100 fold inhibitory selectivity for Edg-1 relative to other Edg receptors.

[0114] In another embodiment, the modulator exhibits at least about 200 fold inhibitory selectivity for Edg-1 relative to other Edg receptors.

[0115] In still another embodiment, the modulator exhibits at least about 200 fold inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0116] In still another embodiment, the modulator exhibits at least about 5 fold 25 inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0117] In still another embodiment, the modulator exhibits at least about 20 fold 25 inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0118] In still another embodiment, the modulator exhibits at least about 100 fold inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0119] In a preferred embodiment, an modulator of cell proliferation exhibits at least about 5 fold inhibitory selectivity for Edg-1 relative to other Edg receptors.

[0120] In another embodiment, the modulator of cell proliferation exhibits at least about 20 fold inhibitory selectivity for Edg-1 relative to other Edg receptors.

[0121] In still another embodiment, the modulator of cell proliferation exhibits at least about 5 fold inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0122] In still another embodiment, the modulator of cell proliferation exhibits at least about 20 fold inhibitory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0123] In other embodiments, the modulator exhibits stimulatory selectivity for the Edg-1 receptor. For example, the modulator exhibits at least about 5 fold stimulatory selectivity for Edg-1 relative to other Edg receptors. Stimulatory selectivity can be measured by assays such as a calcium mobilization assay or a migration and/or invasion assay or a proliferation assay, for example, as described in Sections 7.10, 7.12 and 7.13, respectively. Other assays suitable for determining stimulatory selectivity would be known to one of skill in the art. Preferred assays include the calcium mobilization assay of Section 7.10.

[0124] In another embodiment, the modulator exhibits at least about 20 fold stimulatory selectivity for Edg-1 relative to other Edg receptors.

[0125] In another embodiment, the modulator exhibits at least about 100 fold stimulatory selectivity for Edg-1 relative to other Edg receptors.

[0126] In another embodiment, the modulator exhibits at least about 200 fold stimulatory selectivity for Edg-1 relative to other Edg receptors.

[0127] In still another embodiment, the modulator exhibits at least about 200 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0128] In still another embodiment, the modulator exhibits at least about 5 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0129] In still another embodiment, the modulator exhibits at least about 20 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0130] In still another embodiment, the modulator exhibits at least about 100 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0131] In a preferred embodiment, a modulator of cell proliferation exhibits at least about 5 fold stimulatory selectivity for Edg-1 relative to other Edg receptors.

[0132] In another embodiment, the modulator of cell proliferation exhibits at least about 20 fold stimulatory selectivity for Edg-1 relative to other Edg receptors.

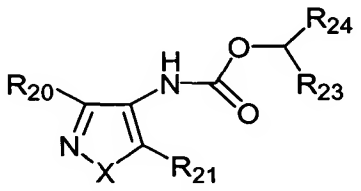
[0133] In still another embodiment, the modulator of cell proliferation exhibits at least about 5 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0134] In still another embodiment, the modulator of cell proliferation exhibits at least about 20 fold stimulatory selectivity for Edg-1 relative to Edg-3, Edg-5, Edg-6 and Edg-8 receptors.

[0135] In one embodiment, the Edg-1 modulator is not a lipid. Preferably, the modulator of Edg-1 receptor mediated biological activity does not contain a phosphate group such as a phosphoric acid, a cyclic phosphate ester or a linear phosphate ester. More preferably, the modulator of the Edg-1 receptor is not a phospholipid. The term "phospholipid" includes all phosphate (both phosphate esters and phosphoric acids) containing glycerol derivatives with an alkyl chain of greater 10 carbon atoms or greater, any N-acyl ethanolamide phosphate derivative (both phosphate esters and phosphoric acids), LPA, S1P or any of their analogues (both phosphate esters and phosphoric acids) (see, *e.g.*, Bandoh, *et al.*, **2000**, *FEBS Lett.* 428, 759; Bittman et al., **1996**, *J. Lipid Research* 391; Lilliom *et al.*, **1996**, *Molecular Pharmacology* 616, Hooks *et al.*, **1998**, *Molecular Pharmacology* 188; Fischer *et al.*, **1998**, *Molecular Pharmacology* 979; Heise *et al.*, **2001**, *Molecular Pharmacology*

1173; Hopper *et al.*, **1999**, *J. Med. Chem.* 42 (6):963-970; Tigyi *et al.*, **2001**, *Molecular Pharmacology* 1161). In certain embodiments, the modulator of the Edg-1 receptor is not sphingosine-1-phosphate, a derivative or analog of sphingosine-1-phosphate or any modulator of Edg-1 activity described in WO 01/69252 or in WO 02/17899 (the contents of each are hereby incorporated by reference in their entireties). In other embodiments, the modulator of the Edg-1 receptor is not FTY720 or any modulator of Edg-1 activity described in Brinkmann *et al.*, 2002, *J. Biol. Chem.* 277: 21453-21457.

[0136] In another embodiment, the modulator is also not a compound of structural formula (IV):



(IV)

or a pharmaceutically available salt thereof, wherein:

X is O or S;

R_{20} is alkyl, substituted alkyl, aryl, substituted aryl or halo;

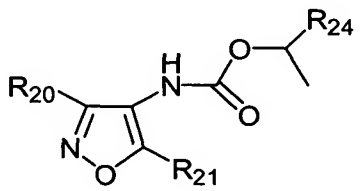
R_{21} is alkyl, substituted alkyl, aryl, substituted aryl, heteroaryl or substituted heteroaryl;

R_{23} is hydrogen, alkyl or substituted alkyl;

R_{24} is aryl, substituted aryl, heteroaryl or substituted heteroaryl;

or alternatively R_{23} and R_{24} form a cycloalkyl ring (International Application No: WO 01/60819).

[0137] In another embodiment, the modulator is not any compound of the formula below:



wherein R_{20}, R_{21} and R_{24} are as previously defined. In yet another embodiment the modulator is not any compound disclosed in International Application No: WO 01/60819 (the content of which is hereby incorporated by reference in its entirety).

[0138] In one preferred embodiment, the modulator is a agonist of the Edg-1 receptor. The modulator can be a weaker agonist than the natural agonist and may compete with the natural agonist for the binding site. In another preferred embodiment, the modulator is antagonist of the Edg-1 receptor. The Edg-1 modulator may be a biomolecule such as a nucleic acid, protein, (*i.e.*, an enzyme or an antibody) or oligosaccharide or any combination thereof. Alternatively, the Edg-1 modulator may be oligomers or monomers of the above biomolecules such as amino acids, peptides, monosaccharides, disaccharides, nucleic acid monomers, dimers, *etc.*, or any combination thereof. The Edg-1 modulator may also be a synthetic polymer or any combination of synthetic polymer with biomolecules including monomers or oligomers of biomolecules.

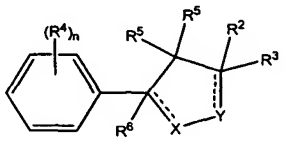
[0139] The Edg-1 modulator may also be an organic molecule of molecular weight less than 1000 daltons. In one embodiment, the molecular weight is about 200 to about 1000 daltons. In another embodiment, the molecular weight is about 200 to about 750 daltons. In yet another embodiment, the molecular weight is about 200 to about 600 daltons. Preferably, the molecular weight is about 300 to about 500 daltons.

[0140] Without wishing to be bound by any particular theory or understanding, the modulator may, for example, facilitate inhibition of the Edg-1 receptor through direct binding to the S1P binding site of the receptor, binding at some other site of the Edg-1 receptor, interference with Edg-1 or S1P biosynthesis, covalent modification of either S1P or the Edg-1 receptor, or may otherwise interfere with Edg-1 mediated signal transduction.

[0141] In one embodiment, the modulator binds to the Edg-1 receptor with a binding constant between about 10 μM and about 1 fM. In another embodiment, the modulator binds to the Edg-1 receptor with a binding constant between about 10 μM and about 1 nM. In another embodiment, the modulator binds to the Edg-1 receptor with a binding constant between about 1 μM and about 1 nM. In another embodiment, the modulator binds to the Edg-1 receptor with a binding constant between about 100 nM and about 1 nM. In another embodiment, the modulator binds

to the Edg-1 receptor with a binding constant between about 10 nM and about 1 nM. Preferably, the modulator binds to the Edg-1 receptor with a binding constant better (*i.e.*, less) than about 10 nM.

[0142] In one aspect, the modulator is a compound of structural formula (I):



(I)

or a pharmaceutically available solvate or hydrate thereof, wherein:

each dashed line indicates an optional double bond;

n = 0, 1, 2, 3, 4 or 5;

X is CR^5 or N;

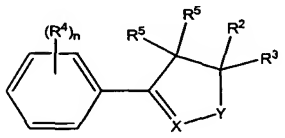
Y is CR^5R^5 or NR^1;

R^1 is or hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylamino, substituted arylamino, arylsulfonyl, substituted arylsulfonyl, carboxy, carbamoyl, substituted carbamoyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, or substituted heteroalkyl;

R^2, R^3 and each R^5 are independently hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylamino, substituted arylamino, arylsulfonyl, substituted arylsulfonyl, carboxy, carbamoyl, substituted carbamoyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio;

each R^4 is independently hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylsulfonyl, substituted arylsulfonyl, azido, carboxy, carbamoyl, substituted carbamoyl, carboxyl, cyano, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, halo, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio; and
R^6 is either absent or hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylamino, substituted arylamino, arylsulfonyl, substituted arylsulfonyl, carboxy, carbamoyl, substituted carbamoyl, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio.

[0143] In certain embodiments, the modulator is a compound of structural formula (Ia):

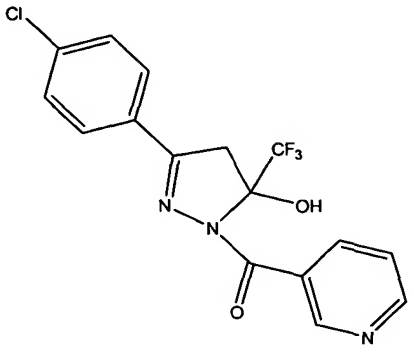


(Ia)

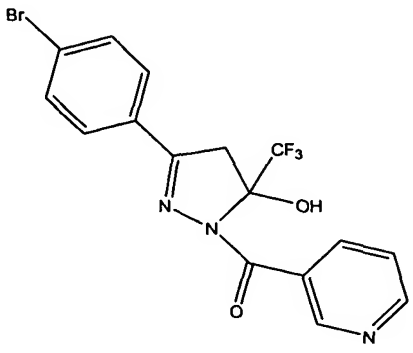
wherein n, X, Y, R^1, R^2, R^3, R^4 and R^5 are as described for structure (I) above.

[0144] In preferred embodiments of the invention, the modulator is a compound of structural formula (I) wherein n is 1; X is N; Y is NR^1, R^1 is acyl or substituted acyl; R^2 is alkyl or substituted alkyl; R^3 is hydrogen, alkoxy, substituted alkoxy, or hydroxyl; each R^5 is H; and R^6 is absent.

[0145] Preferred modulators include **101** and **102**, below, and **105-113** (Table 1). Compounds **105-113** show no activity against Edg receptors other than Edg-1 at 20 μM.



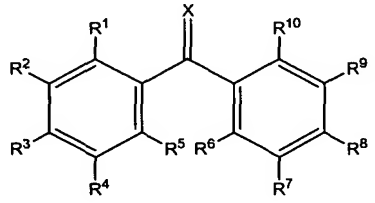
101



102

[0146] In certain embodiments, this aspect of the present invention provides a compound according to structure (I) or (Ia) wherein the compound is not 101, 102 or 105-113.

[0147] In a second aspect of the invention, the modulator is a compound of structural formula (II):



(II)

or a pharmaceutically available solvate or hydrate thereof, wherein:

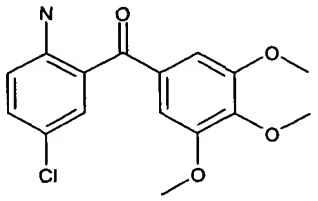
X is O or S; and

each R^1, R^2, R^3, R^4 and R^5 is independently hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylsulfonyl, substituted arylsulfonyl, azido, carboxy, carbamoyl, substituted carbamoyl, carboxyl, cyano, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted

dialkylamino, halo, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio; and each R^6, R^7, R^8, R^9 and R^{10} is independently hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylsulfonyl, substituted arylsulfonyl, azido, carboxy, carbamoyl, substituted carbamoyl, carboxyl, cyano, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, halo, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio.

[0148] In preferred embodiments of the invention, the agonist is a compound of structural formula (II) wherein X is O; each R^1, R^2, R^3, R^4 and R^5 is independently hydrogen, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylarylamino, substituted alkylarylamino, amino, dialkylamino, substituted dialkylamino or halo; and each R^6, R^7, R^8, R^9 and R^{10} is independently hydrogen, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, arylalkyloxy or substituted arylalkyloxy. In further preferred embodiments, the agonist is a compound of structural formula (II) wherein X is O; each R^1, R^2, R^3, R^4 and R^5 is independently hydrogen, amino, or halo; and each R^6, R^7, R^8, R^9 and R^{10} is independently hydrogen, alkoxy or substituted alkoxy. In additional preferred embodiments, the agonist is a compound of structural formula (II) wherein X is O; each R^2, R^3, and R^5 is hydrogen; each R^1 and R^4 is independently amino or halo; each R^6 and R^{10} is hydrogen; and each R^7, R^8, and R^9 is independently alkoxy.

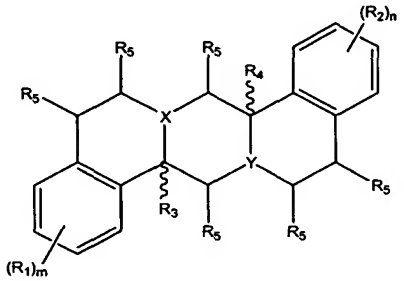
[0149] Preferred modulators also include **104**:



104

[0150] In certain embodiments, this aspect of the present invention provides a compound according to structure (II) wherein the compound is not **104**.

[0151] In a third aspect of the invention, the modulator is a compound of structural formula (III):



(III)

or a pharmaceutically available solvate or hydrate thereof, wherein:

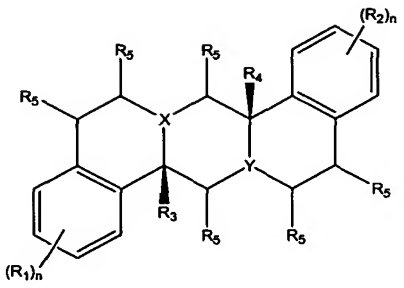
n is 1, 2, 3, 4 or 5;

m is 1, 2, 3, 4, or 5;

each X and Y is independently C or N; and

each R^1, R^2, R^3, R^4 and R^5 is independently hydrogen, alkyl, substituted alkyl, acyl, substituted acyl, acylamino, substituted acylamino, alkylamino, substituted alkylamino, alkylthio, substituted alkylthio, alkoxy, substituted alkoxy, alkoxycarbonyl, substituted alkoxycarbonyl, alkylarylamino, substituted alkylarylamino, arylalkyloxy, substituted arylalkyloxy, amino, aryl, substituted aryl, arylalkyl, substituted arylalkyl, arylsulfonyl, substituted arylsulfonyl, azido, carboxy, carbamoyl, substituted carbamoyl, carboxyl, cyano, cycloalkyl, substituted cycloalkyl, cycloheteroalkyl, substituted cycloheteroalkyl, dialkylamino, substituted dialkylamino, halo, heteroaryloxy, substituted heteroaryloxy, heteroaryl, substituted heteroaryl, heteroalkyl, substituted heteroalkyl, hydroxyl, nitro or thio.

[0152] In a preferred aspect of the invention, the modulator is a compound of structural formula (IIIa):

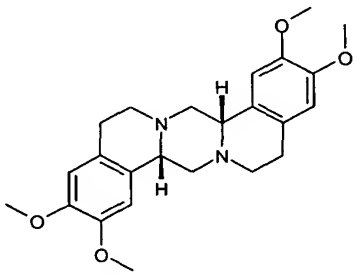


(IIIa)

or a pharmaceutically acceptable salt or hydrate thereof wherein n, m, X, Y, R^1, R^2, R^3, R^4 and R^5 are as defined for structure (III) above.

[0153] In preferred embodiments of the invention, the modulator is a compound of structural formula (III) or (IIIa) wherein n is 2; m is 2; X is N; Y is N; and each R^3, R^4 and R^5 is hydrogen. In further preferred embodiments, the modulator is a compound of structural formula (III) or (IIIa) wherein n is 2; m is 2; X is N; Y is N; and each R^3, R^4 and R^5 is hydrogen; and each R^1 and R^2 is independently alkoxy or substituted alkoxy. In additional preferred embodiments, the modulator is a compound of structural formula (III) or (IIIa) wherein n is 2; m is 2; X is N; Y is N; and each R^3, R^4 and R^5 is hydrogen; and each R^1 and R^2 is methoxy.

[0154] Preferred modulators include **103:**



103

[0155] In certain embodiments, this aspect of the present invention provides a compound according to structure (III) or (IIIa) wherein the compound is not **103**.

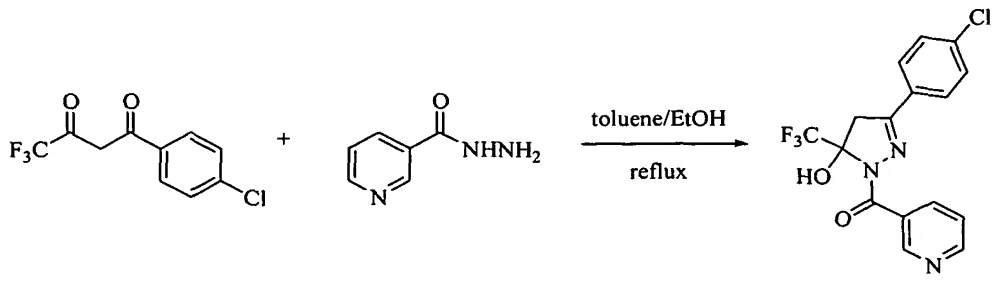
[0156] Finally, in certain embodiments, the Edg-1 receptor modulators can be used in combination with one or more modulators of other Edg receptors. In certain embodiments, the modulators of other Edg receptors can modulate the Edg-2 receptor. In other embodiments, the modulators of other Edg receptors can modulate the Edg-3

receptor. In still other embodiments, the modulators of other Edg receptors can modulate the Edg-4 receptor. In yet other embodiments, the modulators of other Edg receptors can modulate the Edg-7 receptor. Modulators of these other Edg receptors are extenisvely described in co-pending U.S. Patent Application Nos. 10/390,427, 10/390,426, 10/390,429, and 10/390,428, each of which is hereby incorporated by reference in its entirety.

6.3. Synthesis of the Compounds of the Invention

[0157] The compounds of the invention may be obtained *via* the synthetic methods illustrated in Scheme 1. Starting materials useful for preparing compounds of the invention and intermediates thereof are commercially available or can be prepared by well-known synthetic methods. Other methods for synthesis of the compounds described herein are either described in the art or will be readily apparent to the skilled artisan in view of general references well-known in the art (See *e.g.*, Green *et al.*, "Protective Groups in Organic Chemistry", (Wiley, 2nd ed. 1991); Harrison *et al.*, "Compendium of Synthetic Organic Methods", Vols. 1-8 (John Wiley and Sons, 1971-1996); "Beilstein Handbook of Organic Chemistry," Beilstein Institute of Organic Chemistry, Frankfurt, Germany; Feiser *et al.*, "Reagents for Organic Synthesis," Volumes 1-17, Wiley Interscience; Trost *et al.*, "Comprehensive Organic Synthesis," Pergamon Press, 1991; "Theilheimer's Synthetic Methods of Organic Chemistry," Volumes 1-45, Karger, 1991; March, "Advanced Organic Chemistry," Wiley Interscience, 1991; Larock "Comprehensive Organic Transformations," VCH Publishers, 1989; Paquette, "Encyclopedia of Reagents for Organic Synthesis," John Wiley & Sons, 1995) and may be used to synthesize the compounds of the invention. Accordingly, the methods presented in Scheme 1 herein are illustrative rather than comprehensive.

[0158] The route described in Scheme 1 may used to synthesize compounds of Formula (I).



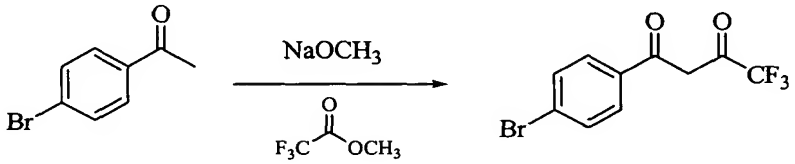
6.3.1. Compound 101

[0159] A solution of 1-(4-chlorophenyl)-4,4,4-trifluoro-1,3-butanedione (0.883 g, 3.17 mmol) and nicotinic acid hydrazide (0.448 g, 3.17 mmol) in ethanol (~2 mL) and toluene (50 mL) was heated at reflux. After 2 days the reaction mixture was reduced *in vacuo* and the crude residue was subjected to flash column chromatography over silica gel (98:2 CH_2Cl_2/MeOH). Recrystallization of the impure product from ethanol afforded 5-(4-chlorophenyl)-2-pyridin-3-ylmethyl-3-trifluoromethyl-3,4-dihydro-2*H*-pyrazol-3-ol (0.134 g, 11% yield) as a light yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.24 (m, 1H), 8.79 (m, 1H), 8.27 (m, 1H), 7.59 (m, 2H), 7.42 (m, 3H), 6.47 (s, 1H), 3.74 (d, 1H), 3.75 (d, 1H). ESI-MS *m/z*: 370 $[C_{16}H_{11}ClF_3N_3O_2 + H]^+$. m.p. 158 –160 °C.

6.3.2. Compound 102

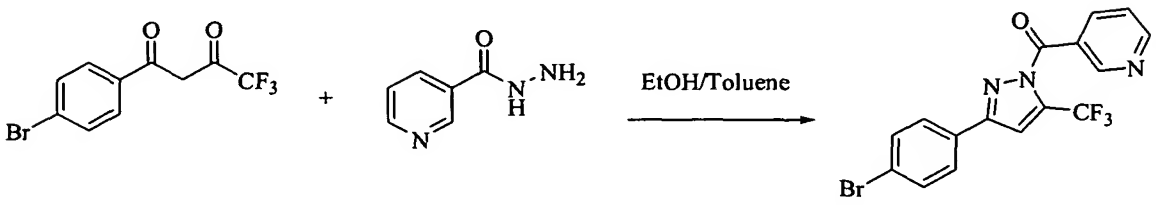
[0160] Compound 102 can be prepared according to the following synthetic scheme.

(a) Synthesis of 1-(4-Bromophenyl)-4,4,4-trifluoro-1,3-butanedione



[0161] To a solution of ethyl trifluoroacetate (81.0 mL, 0.452 mol) in methoxy *tert*-butyl ether (0.5 L) was slowly added sodium methoxide (30 wt % in methanol, 102 mL, 0.542 mol) at room temperature. The reaction exothermed and was cooled to room temperature with an ice bath. 4'-Bromoacetophenone (90.0 g, 0.452 mol) was added portion-wise to the reaction mixture over 10 min. and the reaction mixture was stirred overnight at room temperature. The organic layer was washed with hydrochloric acid (3N, 300 mL), brine (100 mL), dried (magnesium sulfate), filtered, and concentrated *in vacuo*. The residue was recrystallized from hexanes five times to provide 1-(4-bromophenyl)-4,4,4-trifluoro-1,3-butanedione (96.5 g, 72%) as a pale yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.9 (s, 1H), 7.83–7.75 (m, 2H), 7.68–7.60 (m, 2H), 6.54 (s, 1H).

(b) Preparation of [3-(4-Bromophenyl)-5-trifluoromethylpyrazol-1-yl]pyridin-3-yl-methanone



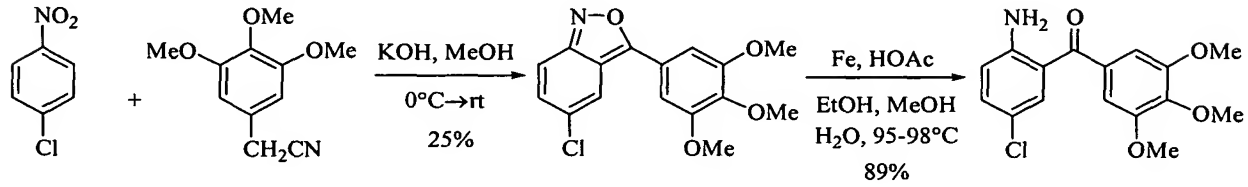
[0162] A mixture of 1-(4-bromophenyl)-4,4,4-trifluoro-1,3-butanedione (55.2 g, 0.187 mol) and nicotinic acid hydrazide (25.6 g, 0.187 mol) in absolute ethanol (0.7 L) was stirred at reflux overnight. The reaction mixture was allowed to cool to room temperature and then was concentrated *in vacuo*. The crude residue was taken up in toluene (0.7 L) and was stirred at reflux for two days. The mixture was allowed to cool and then was concentrated *in vacuo*. The residue was treated with methoxy *tert*-butyl ether (0.4 L) and stirred at 55 °C for thirty min. The solids were filtered off and the mother liquor concentrated *in vacuo*. The crude residue was purified by chromatography (silica gel, 0.5% methanol/methylene chloride) to obtain [3-(4-bromophenyl)-5-trifluoromethylpyrazol-1-yl]pyridin-3-yl-methanone (13.0 g, 17%) as a white solid: mp 150-152 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 9.11–8.92 (m, 1H), 8.69–8.58 (m, 1H), 8.38–8.20 (m, 1H), 7.74–7.42 (m, 5H), 3.95 (d, 1H), 3.60 (d, 1H); ESI MS *m/z* 414 $[C_{16}H_{11}BrF_3N_3O_2 + H]^+$.

[0163] Compound **102** is also commercially available compound from Chembridge (San Diego, CA) #5795726

6.3.3. Compound 103

[0164] Compound **103** is commercially available compound from Specs/BioSpecs(The Netherlands) #AH-262/34399012

6.3.4. Compound 104



[0165] Potassium hydroxide pellets (85%, 5.76g, 87.3 mmol, 17.5 equiv) were dissolved in methanol (12 mL). The mixture was cooled with an ice bath, and a solution of *p*-chloronitrobenzene (788 mg, 5 mmol) and 3,4,5-

trimethoxyphenylacetonitrile (Acros, 1.140 g, 5.5 mmol) in methanol (15 mL) was added dropwise (ref. Davis, R.B.; Pizzini, L.C. *J. Org. Chem.* **1960**, *25*, 1884-1888). The dark purple reaction mixture was slowly warmed up to room temperature overnight and then poured into water (250 mL). The precipitates were collected by a suction filtration, washed several times with water, air-dried, and subject to flash column chromatography over silica gel using ethyl acetate/hexanes (1:10, 1:6, and 1:4). 5-Chloro-3-(3,4,5-trimethoxy-phenyl)benzo[*c*]isoxazole was isolated as a slightly yellow solid (405 mg, 25% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (m, 1H), 7.59 (m, 1H), 7.27 (m, 1H), 7.16 (s, 2H), 4.01 (s, 6H), 3.96 (s, 3H). APCI-MS: *m/z* 320 $[C_{16}H_{14}ClNO_4 + H]^+$. m.p. 119 – 120°C.

[0166] A mixture of iron powder (183 mg, 3.28 mmol, 3 equiv), ethanol (4 mL), water (2 mL), and acetic acid (6 mL) was preheated to 60°C (oil bath). 5-Chloro-3-(3,4,5-trimethoxyphenyl)-benzo[*c*]isoxazole (350 mg, 1.10 mmol) in methanol (10 mL) was then added and the reaction mixture was stirred for 30 min at that temperature and then overnight at 95-98°C. Most of the solvents were removed by rotary evaporation and the residue was diluted with dichloromethane (100 mL). Insoluble inorganics were removed by filtration and the filtrate solution was concentrated by rotary evaporation. The resulting solid was subject to flash column chromatography over silica gel using ethyl acetate/hexanes (1:10, 1:6, 1:4, and 1:2). (2-Amino-5-chlorophenyl)-(3,4,5-trimethoxyphenyl)methanone was isolated as a yellow solid (315 mg, 89% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 7.35 (m, 1H), 7.24 (m, 1H), 6.91 (s, 2H), 6.83 (m, 1H), 3.86 (s, 6H), 3.85 (s, 3H). APCI-MS: *m/z* 322 $[C_{16}H_{16}ClNO_4 + H]^+$. m.p. 141 – 142°C.

6.4. Therapeutic Uses of the Compounds of the Invention

[0167] The compounds and/or compositions of the present invention may be used to treat diseases, including but not limited to, ovarian cancer (Xu *et al.*, **1995**, *Biochem. J.* 309 (Pt 3):933-940; Xu *et al.*, **1998**, *JAMA* 280 (8):719-723; Goetzl *et al.*, **1999**, *Cancer Res.* 59 (20):5370-5375), peritoneal cancer, endometrial cancer, cervical cancer, breast cancer, colorectal cancer, uterine cancer, stomach cancer, small intestine cancer, thyroid cancer, lung cancer, kidney cancer, pancreas cancer and prostrate cancer; acute lung diseases, adult respiratory distress syndrome ("ARDS"), acute inflammatory exacerbation of chronic lung diseases such as asthma (Chilton *et al.*, **1996**, *J Exp Med* 183:2235-45; Arbibe *et al.*, **1998**, *J Clin Invest* 102:1152-60)

surface epithelial cell injury, (*e.g.*, transcorneal freezing or cutaneous burns (Liliom *et al.*, **1998**, *Am. J. Physiol* 274 (4 Pt 1): C1065-C1074)), cardiovascular diseases, (*e.g.*, ischemia (Karliner *et al.*, **2001**, *J. Mol. Cell Cardiol.* 33 (9):1713-1717) and atherosclerosis (Siess *et al.*, **1999**, *Proc. Natl. Acad. Sci. U.S.A.* 96 (12):6931-6936; Siess *et al.*, **2000**, *IUBMB Life* 49 (3):167-171)).

[0168] In accordance with the invention, a compound and/or composition of the invention is administered to a subject, preferably a human, in need of treatment for a disease which includes but is not limited to, the diseases listed above. Further, in certain embodiments, the compounds and/or compositions of the invention can be administered to a subject, preferably a human, as a preventative measure against diseases or disorders such as those depicted above. Thus, the compounds and/or compositions of the invention can be administered as a preventative measure to a subject having a predisposition, which includes but is not limited to, the diseases listed above. Accordingly, the compounds and/or compositions of the invention may be used for the prevention of one disease or disorder and concurrently treating another disease (*e.g.*, preventing cancer and treating cardiovascular diseases).

[0169] In another aspect, the compounds and/or compositions of the invention can be used to treat or prevent disorders involving an immune response. One of skill in the art will readily recognize which disorders can be treated or prevented by stimulating or potentiating an immune response and which disorders can be treated or preventing by suppressing an immune response.

[0170] Where the immune response is to be stimulated, the Edg-1 receptor modulators are preferably, but not limited to, Edg-1 receptor agonists. One of ordinary skill in the art can readily determine whether a particular Edg-1 agonist or antagonist can stimulate an immune response by performing, for example, a migration and invasion assay as described in Section 7.12 or a migration assay in the mouse dorsal air-pouch model as described in Section 7.9. Further, one of ordinary skill in the art can readily identify whether a subject will benefit from stimulation of an immune response. For example, subjects that suffer from an inherited immune deficiency will benefit from stimulation of an immune response. Other such subjects include subjects infected with a virus, including, but not limited to, cytomegalovirus, herpes simplex virus I, herpes simplex virus II, influenza A virus, influenza B virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. In yet other embodiments, the subject that will benefit from stimulation of an

immune response include subjects that are administered a vaccine. In such embodiments, an Edg-1 receptor modulator can be administered as an adjuvant to a vaccine.

[0171] In other embodiments, the immune response can be suppressed by Edg-1 receptor modulators. In such embodiments, the Edg-1 receptor modulators can be Edg-1 receptor agonists or antagonists, as described above. One of ordinary skill in the art can readily determine whether a particular Edg-1 agonist or antagonist can suppress an immune response by performing, for example, a migration and invasion assay as described in Section 7.12 or a migration assay in the mouse dorsal air-pouch model as described in Section 7.9. In addition, one of skill in the art can readily recognize subjects that will benefit from suppression of an immune response. For example, such subjects include those that suffer from an immune disorder that is characterized by an inappropriate activation of the immune system.

[0172] Such disorders include, but are not limited to, systemic lupus erythematosus, rheumatic cardiatis, polymyosis, pemphigus, bullous dermatits herpetiformis, Stevens-Johnson syndrome, mycosis fungoides, dermatitis, ulcerative colitis, Crohn's disease, intractable sprue, idiopathic thrombocytopenic purpura, hemolytic anemia, erythroblastopenia, congenital hypoplastic anemia, osteoarthritis, rheumatoid arthritis, bursitis, acute gouty arthritis, epicondylitis, acute nonspecific tenosynovitis, multiple sclerosis, keratitis, irititis, irisocyclitis, chorioretinitis, choroiditis, optic neuritis, sarcoiodosis, Loeffler's syndrome, berylliosis, tuberculosis, sponylitis, tenosynovitis, psoriatic arthritis, and type I diabetes mellitus. Another example of a subject that will benefit from suppression of an immune response is a subject that is the recipient of a transplanted cell, tissue, or organ.

[0173] One of ordinary skill in the art can readily determine whether a particular Edg-1 agonist or antagonist has successfully stimulated or suppressed an immune response when administered to a subject. For example, a delayed-type hypersensitivity assay can be performed on the subject to test the subject's cell-based immunity. In such assays, an antigen to which the subject has previously been exposed is injected subcutaneously. If the subject's immune response has been suppressed, no delayed-type hypersensitivity or mild delayed-type hypersensitivity is observed. If the subject's immune response has not been suppressed, a more robust delayed-type hypersensitivity reaction is observed. If the subject's immune response has been stimulated, no delayed-type hypersensitivity or mild delayed-type

hypersensitivity is observed. Delayed-type hypersensitivity assays are well-known to the art and can readily be performed by one of ordinary skill.

[0174] In a preferred embodiment, Edg-1 antagonists can be used to treat or prevent disorders such as, but not limited to, vasoconstriction in cerebral arteries, autoimmune and related immune disorders, including, but not limited to, systemic lupus erythematosus, rheumatoid arthritis, non-glomerular nephrosis, psoriasis, chronic active hepatitis, ulcerative colitis, Crohn's disease, Behçet's disease, chronic glomerulonephritis, chronic thrombocytopenic purpura, and autoimmune hemolytic anemia. Additionally, Edg-1 agonists and/or antagonists can also be used as immunosuppressants in organ transplantation, as well as inhibitors of angiogenesis for treatment of various cancers. In yet another embodiment, Edg-1 agonists and antagonists can be used to treat vascular occlusive disorders. For example, activation of Edg-1 receptors by using an Edg-1 agonist will result in increased vasoconstriction which is beneficial in conditions such as migraine headaches. Inhibition of Edg-1 by an Edg-1 antagonist will be beneficial in conditions such as a stroke, a subarachnoid hemorrhage, or a vasospasm such as a cerebral vasospasm. It is well within the capability of those of skill in the art to assay and use the compounds and/or compositions of the invention to treat diseases, such as the diseases listed above.

6.5. Therapeutic/Prophylactic Administration

[0175] The compounds and/or compositions of the invention may be advantageously used in medicine, including human medicine. As previously described in Section 6.4 above, compounds and compositions of the invention are useful for the treatment or prevention of diseases, which include but are not limited to, cancers, including, but not limited to, ovarian cancer, peritoneal cancer, endometrial cancer, cervical cancer, breast cancer, colorectal cancer, uterine cancer, stomach cancer, small intestine cancer, thyroid cancer, lung cancer, kidney cancer, pancreas cancer, prostrate cancer, acute lung diseases, including, but not limited to, adult respiratory distress syndrome (ARDS) and acute inflammatory exacerbation of chronic lung diseases such as asthma; surface epithelial cell injury, including, but not limited to, transcorneal freezing or cutaneous burns; cardiovascular diseases, including, but not limited to, ischemia and atherosclerosis; viral infections, including, but not limited to, cytomegalovirus, herpes simplex virus I, herpes simplex virus II, influenza A virus, influenza B virus, hepatitis A virus, hepatitis B virus, hepatitis C

virus, and human immunodeficiency virus infections; and immune disorders, including, but not limited to, systemic lupus erythematosus, rheumatic cardiatis, polymyosis, pemphigus, bullous dermatits herpetiformis, Stevens-Johnson syndrome, mycosis fungoides, dermatitis, ulcerative colitis, Crohn's disease, intractable sprue, idiopathic thrombocytopenic purpura, hemolytic anemia, erythroblastopenia, congenital hypoplastic anemia, osteoarthritis, rheumatoid arthritis, bursitis, acute gouty arthritis, epicondylitis, acute nonspecific tenosynovitis, multiple sclerosis, keratitis, irititis, irisocyclitis, chorioretinitis, choroiditis, optic neuritis, sarcoiodosis, Loeffler's syndrome, berylliosis, tuberculosis, sponylitis, tenosynovitis, psoriatic arthritis, and type I diabetes mellitus.

[0176] In a preferred embodiment, Edg-1 antagonists can be used to treat or prevent disorders such as, but not limited to, vasoconstriction in cerebral arteries, autoimmune and related immune disorders, including, but not limited to, systemic lupus erythematosus, rheumatoid arthritis, non-glomerular nephrosis, psoriasis, chronic active hepatitis, ulcerative colitis, Crohn's disease, Behçet's disease, chronic glomerulonephritis, chronic thrombocytopenic purpura, and autoimmune hemolytic anemia. Additionally, Edg-1 agonists and/or antagonists can also be used as immunosuppressants in organ transplantation, as well as inhibitors of angiogenesis for treatment of various cancers.. In yet another embodiment, Edg-1 agonists and antagonists can be used to treat vascular occlusive disorders. For example, activation of Edg-1 receptors by using an Edg-1 agonist will result in increased vasoconstriction which is beneficial in conditions such as migraine headaches. Inhibition of Edg-1 by an Edg-1 antagonist will be beneficial in conditions such as a stroke, a subarachnoid hemorrhage, or a vasospasm such as a cerebral vasospasm.

[0177] When used to treat or prevent disease or disorders, compounds and/or compositions of the invention may be administered or applied singly, in combination with other agents. The compounds and/or compositions of the invention may also be administered or applied singly, in combination with other pharmaceutically active agents, including other compounds and/or compositions of the invention.

[0178] The current invention provides methods of treatment and prophylaxis by administration to a subject of a therapeutically effective amount of a composition or compound of the invention. The subject may be an animal, is more preferably a mammal, and most preferably a human.

[0179] The present compounds and/or compositions of the invention, which comprise one or more compounds of the invention, are preferably administered orally. The compounds and/or compositions of the invention may also be administered by any other convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*). Administration can be systemic or local. Various delivery systems are known, (*e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*) that can be used to administer a compound and/or composition of the invention. Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. The preferred mode of administration is left to the discretion of the practitioner, and will depend in-part upon the site of the medical condition. In most instances, administration will result in the release of the compounds and/or compositions of the invention into the bloodstream.

[0180] In specific embodiments, it may be desirable to administer one or more compounds and/or composition of the invention locally to the area in need of treatment. This may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of the diseases listed above.

[0181] In certain embodiments, it may be desirable to introduce one or more compounds and/or compositions of the invention into the central nervous system by any suitable route, including intraventricular, intrathecal and epidural injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

[0182] A compound and/or composition of the invention may also be administered directly to the lung by inhalation. For administration by inhalation, a compound and/or composition of the invention may be conveniently delivered to the lung by a number of different devices. For example, a Metered Dose Inhaler ("MDI"), which utilizes canisters that contain a suitable low boiling propellant, (*e.g.*,

dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or any other suitable gas) may be used to deliver compounds of the invention directly to the lung.

[0183] Alternatively, a Dry Powder Inhaler ("DPI") device may be used to administer a compound and/or composition of the invention to the lung. DPI devices typically use a mechanism such as a burst of gas to create a cloud of dry powder inside a container, which may then be inhaled by the subject. DPI devices are also well known in the art. A popular variation is the multiple dose DPI ("MDDPI") system, which allows for the delivery of more than one therapeutic dose. For example, capsules and cartridges of gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of a compound of the invention and a suitable powder base such as lactose or starch for these systems.

[0184] Another type of device that may be used to deliver a compound and/or a composition of the invention to the lung is a liquid spray device. Liquid spray systems use extremely small nozzle holes to aerosolize liquid drug formulations that may then be directly inhaled into the lung.

[0185] In one embodiment, a nebulizer is used to deliver a compound and/or composition of the invention to the lung. Nebulizers create aerosols from liquid drug formulations by using, for example, ultrasonic energy to form fine particles that may be readily inhaled (see *e.g.*, Verschoyle *et al.*, *British J. Cancer* **1999**, 80, Suppl. 2, 96, which is herein incorporated by reference). Examples of nebulizers include devices supplied by Sheffield/Systemic Pulmonary Delivery Ltd. (See, Armer *et al.*, United States Patent No. 5,954,047; van der Linden *et al.*, United States Patent No. 5,950,619; van der Linden *et al.*, United States Patent No. 5,970,974), Aventis and Batelle Pulmonary Therapeutics.

[0186] In another embodiment, an electrohydrodynamic ("EHD") aerosol device is used to deliver a compound and/or composition of the invention to the lung. EHD aerosol devices use electrical energy to aerosolize liquid drug solutions or suspensions (see *e.g.*, Noakes *et al.*, United States Patent No. 4,765,539). EHD aerosol devices may more efficiently deliver drugs to the lung than other pulmonary delivery technologies.

[0187] In another embodiment, the compounds of the invention can be delivered in a vesicle, in particular a liposome (*see* Langer, *Science* **1990**, 249:1527-1533; Treat *et al,* in "Liposomes in the Therapy of Infectious Disease and Cancer,"

Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); *see* generally "Liposomes in the Therapy of Infectious Disease and Cancer," Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989)).

[0188] In yet another embodiment, the compounds of the invention can be delivered *via* sustained release systems, preferably oral sustained release systems. In one embodiment, a pump may be used (*see* Langer, *supra;* Sefton, 1987, *CRC Crit Ref Biomed. Eng.* 14:201; Saudek *et al., N. Engl. J Med.* **1989**, 321:574).

[0189] In another embodiment, polymeric materials can be used (*see* "Medical Applications of Controlled Release," Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); "Controlled Drug Bioavailability," Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol Chem.* **1983**, 23:61; *see also* Levy *et al., Science* **1985**, 228: 190; During *et al., Ann. Neurol.* **1989**, 25:351; Howard *et al, J. Neurosurg.* **1989**, 71:105). In a preferred embodiment, polymeric materials are used for oral sustained release delivery. In another embodiment, enteric-coated preparations can be used for oral sustained release administration. In still another embodiment, osmotic delivery systems are used for oral sustained release administration (Verma *et al., Drug Dev. Ind. Pharm.* **2000**, 26:695-708).

[0190] In yet another embodiment, a controlled-release system can be placed in proximity of the target of the compounds and/or composition of the invention, thus requiring only a fraction of the systemic dose *(see, e.g.,* Goodson, in "Medical Applications of Controlled Release," *supra,* vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in Langer, **1990**, *Science* 249:1527-1533 may also be used.

6.6. Compositions of the Invention

[0191] The present compositions contain a therapeutically effective amount of one or more compounds of the invention, preferably in purified form, together with a suitable amount of a pharmaceutically acceptable vehicle, so as to provide the form for proper administration to a subject. When administered to a subject, the compounds of the invention and pharmaceutically acceptable vehicles are preferably sterile. Water is a preferred vehicle when the compound of the invention is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid vehicles, particularly for injectable solutions.

Suitable pharmaceutical vehicles also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents or pH buffering agents. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents may be used.

[0192] Pharmaceutical compositions comprising a compound of the invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries, which facilitate processing of compounds of the invention into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

[0193] The present compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable vehicle is a capsule (see *e.g.*, Grosswald *et al.*, United States Patent No. 5,698,155). Other examples of suitable pharmaceutical vehicles have been described in the art (see Remington's Pharmaceutical Sciences, Philadelphia College of Pharmacy and Science, 17th Edition, 1985).

[0194] For topical administration compounds of the invention may be formulated as solutions, gels, ointments, creams, suspensions, *etc.* as are well-known in the art.

[0195] Systemic formulations include those designed for administration by injection, *e.g.*, subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, oral or pulmonary administration. Systemic formulations may be made in combination with a further active agent that improves mucociliary clearance of airway mucus or reduces mucous viscosity. These active agents include, but are not limited to, sodium channel blockers, antibiotics, N-acetyl cysteine, homocysteine and phospholipids.

[0196] In a preferred embodiment, the compounds of the invention are formulated in accordance with routine procedures as a composition adapted for

intravenous administration to human beings. Typically, compounds of the invention for intravenous administration are solutions in sterile isotonic aqueous buffer. For injection, a compound of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. When necessary, the compositions may also include a solubilizing agent. Compositions for intravenous administration may optionally include a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. When the compound of the invention is administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. When the compound of the invention is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0197] For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0198] Compositions for oral delivery may be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions may contain one or more optionally agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry coloring agents and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions may be coated to delay disintegration and absorption in the gastrointestinal tract, thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compounds of the invention. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the

spiked profiles of immediate release formulations. A time delay material such as glycerol monostearate or glycerol stearate may also be used. Oral compositions can include standard vehicles such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Such vehicles are preferably of pharmaceutical grade.

[0199] For oral liquid preparations such as, for example, suspensions, elixirs and solutions, suitable carriers, excipients or diluents include water, saline, alkyleneglycols (*e.g.*, propylene glycol), polyalkylene glycols (*e.g.*, polyethylene glycol) oils, alcohols, slightly acidic buffers between pH 4 and pH 6 (*e.g.*, acetate, citrate, ascorbate at between about 5.0 mM to about 50.0 mM, *etc*). Additionally, flavoring agents, preservatives, coloring agents, bile salts, acylcarnitines and the like may be added.

[0200] For buccal administration, the compositions may take the form of tablets, lozenges, *etc.* formulated in conventional manner.

[0201] Liquid drug formulations suitable for use with nebulizers and liquid spray devices and EHD aerosol devices will typically include a compound of the invention with a pharmaceutically acceptable vehicle. Preferably, the pharmaceutically acceptable vehicle is a liquid such as alcohol, water, polyethylene glycol or a perfluorocarbon. Optionally, another material may be added to alter the aerosol properties of the solution or suspension of compounds of the invention. Preferably, this material is liquid such as an alcohol, glycol, polyglycol or a fatty acid. Other methods of formulating liquid drug solutions or suspension suitable for use in aerosol devices are known to those of skill in the art (see, *e.g.*, Biesalski, United States Patent No. 5,112,598; Biesalski, United States Patent No. 5,556,611).

[0202] A compound of the invention may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

[0203] In addition to the formulations described previously, a compound of the invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (*e.g.*, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, a compound of the invention may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0204] When a compound of the invention is acidic, it may be included in any of the above-described formulations as the free acid, a pharmaceutically acceptable salt, a solvate or hydrate. Pharmaceutically acceptable salts substantially retain the activity of the free acid, may be prepared by reaction with bases and tend to be more soluble in aqueous and other protic solvents than the corresponding free acid form.

6.7. Methods of Use And Doses

[0205] A compound of the invention, or compositions thereof, will generally be used in an amount effective to achieve the intended purpose. The compounds of the invention or compositions thereof, are administered or applied in a therapeutically effective amount for use to treat or prevent diseases or disorders including but not limited to, ovarian cancer, peritoneal cancer, endometrial cancer, cervical cancer, breast cancer, colorectal cancer, uterine cancer, stomach cancer, small intestine cancer, thyroid cancer, lung cancer, kidney cancer, pancreas cancer, prostrate cancer, acute lung diseases, (*e.g.*, adult respiratory distress syndrome (ARDS) and asthma) surface epithelial cell injury (*e.g.*, transcorneal freezing and cutaneous burns) and cardiovascular diseases such as ischemia and atherosclerosis.

[0206] The amount of a compound of the invention that will be effective in the treatment of a particular disorder or condition disclosed herein will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques known in the art as previously described. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The amount of a compound of the invention administered will, of course, be dependent on, among other factors, the subject being treated, the weight of the subject, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

[0207] For example, the dosage may be delivered in a pharmaceutical composition by a single administration, by multiple applications or controlled release. In a preferred embodiment, the compounds of the invention are delivered by oral sustained release administration. Preferably, in this embodiment, the compounds of the invention are administered twice per day (more preferably, once per day). Dosing may be repeated intermittently, may be provided alone or in combination with other drugs and may continue as long as required for effective treatment of the disease state or disorder.

[0208] Suitable dosage ranges for oral administration are dependent on the potency of the, but are generally about 0.001 mg to about 200 mg of a compound of the invention per kilogram body weight. Dosage ranges may be readily determined by methods known to the skilled artisan.

[0209] Suitable dosage ranges for intravenous (i.v.) administration are about 0.01 mg to about 100 mg per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 mg/kg body weight to about 1 mg/kg body weight. Suppositories generally contain about 0.01 milligram to about 50 milligrams of a compound of the invention per kilogram body weight and comprise active ingredient in the range of about 0.5% to about 10% by weight. Recommended dosages for intradermal, intramuscular, intraperitoneal, subcutaneous, epidural, sublingual or intracerebral administration are in the range of about 0.001 mg to about 200 mg per kilogram of body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Such animal models and systems are well known in the art.

[0210] The compounds of the invention are preferably assayed *in vitro* and *in vivo,* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays can be used to determine whether administration of a specific compound of the invention or a combination of compounds of the invention is preferred for reducing convulsion. The compounds of the invention may also be demonstrated to be effective and safe using animal model systems.

[0211] Preferably, a therapeutically effective dose of a compound of the invention described herein will provide therapeutic benefit without causing substantial toxicity. Toxicity of compounds of the invention may be determined using standard pharmaceutical procedures and may be readily ascertained by the skilled artisan. The dose ratio between toxic and therapeutic effect is the therapeutic index. A compound of the invention will preferably exhibit particularly high therapeutic indices in treating disease and disorders. The dosage of a compound of the inventions described herein will preferably be within a range of circulating concentrations that include an effective dose with little or no toxicity.

6.8. Combination Therapy

[0212] In certain embodiments, the compounds of the invention can be used in combination therapy with at least one other therapeutic agent. The compound of the

invention and the other therapeutic agent can act additively or, more preferably, synergistically. In a preferred embodiment, a compound of the invention is administered concurrently with the administration of another therapeutic agent. In another preferred embodiment, a composition comprising a compound of the invention is administered concurrently with the administration of another therapeutic agent, which can be part of the same composition as the compound of the invention or a different composition. In another embodiment, a composition comprising a compound of the invention is administered prior or subsequent to administration of another therapeutic agent. Other therapeutic agents, which may be used with the compounds and/or compositions of the invention, include but are not limited to, agonists and antagonists of Edg-1, drugs used to treat cardiovascular diseases and/or cancer such as, alkylating agents (*e.g.*, cyclophosphamide, melphalan, chlorambucil), platinum compounds (*e.g.*, cisplatin, carboplatin), anthracyclines (*e.g.*, doxorubicin, epirubicin), taxanes (*e.g.*, paclitaxel, docetaxel), chronic oral etoposide, topotecan, gemcitabine, hexamethylamine, methotrexate, and 5-fluorouracil.

6.9. Assays

[0213] One of skill in the art can use the following assays to identify Edg-1 agonists or antagonists.

6.9.1. Intracellular Calcium Measurement Assays

[0214] Specific assays for Edg-1 receptor activity are known to those of skill in the art. For example, cells expressing Edg-1 receptors can be contacted with a membrane-permeant calcium sensitive dye such as Fluo-4 AM or a proprietary calcium dye loading kit (*e.g.*, FLIPR Calcium Assay kit, Molecular Devices, Sunnyvale, CA). Intracellular calcium is capable of binding to the dye and emitting fluorescent radiation when illuminated at the appropriate wavelength. The cells can thus be illuminated an appropriate wavelength for the dye and any emitting light can be captured by a cooled CCD camera. Changes in fluorescence indicate changes in intracellular calcium resulting from the activation of an Edg-1 receptor. Such changes can be measured advantageously in whole cells in "real-time" (Berridge *et al.*, Nature Reviews 2000, 1:11-21). Exemplary methods are described in co-pending U.S. application no. U.S. application no. 09/904,099, filed July 11, 2001, the content of which is hereby incorporated by reference in its entirety.

[0215] Other methods of measuring intracellular calcium are known to those of skill in the art. For instance, a commonly used technique is the expression of receptors of interest in *Xenopus laevis* oocytes followed by measurement of calcium activated chloride currents (see Weber, 1999, *Biochim Biophys Acta* 1421:213-233). In addition, several calcium sensitive dyes are available for the measurement of intracellular calcium. Such dyes can be membrane permeant or not membrane permeant. Examples of useful membrane permeant dyes include acetoxymethyl ester forms of dyes that can be cleaved by intracellular esterases to form a free acid, which is no longer membrane permeant and remains trapped inside a cell. Dyes that are not membrane permeant can be introduced into the cell by microinjection, chemical permeabilization, scrape loading and similar techniques (Haughland, 1993, in "Fluorescent and Luminescent Probes for Biological Activity" ed. Mason, W.T. pp 34-43; Academic Press, London; Haughland, 1996, in "Handbook of Fluorescent Probes and Research Chemicals", sixth edition, Molecular Probes, Eugene, OR).

6.9.2. IL-8 and VEGF Assays

[0216] The levels of interleukin-8 ("IL-8") and vascular endothelial growth factor ("VEGF") are important markers for the proliferative potential, angiogenic capacity and metastatic potential of a tumor cell line. Specific assays for IL-8 and VEGF are known to those of skill in the art. For example, IL-8 and VEGF assays can be performed by techniques that include, but are not limited to, a standard enzyme-linked immunosorbent assay ("ELISA"). In a standard ELISA, the cells can be cultured, for example, in a 96 well format, serum starved overnight, and treated with LPA or S1P. Dose ranges would be known to one of skill in the art. For example, the doses can range from 0.1-10 μM in serum free medium. Cell supernatants can then be collected to measure the amount of IL-8 or VEGF secreted.

[0217] Methods to measure the amount of IL-8 or VEGF secreted are known to one of skill in the art. In one method, an anti-IL-8 or anti-VEGF capture antibody can be adsorbed on to any surface, for example, a plastic dish. Cell supernatants containing IL-8 or VEGF can then be added to the dish and any method known in the art for detecting antibodies can be used to detect the anti-IL-8 or anti-VEGF antibody. In one embodiment, an anti-IL-8 or anti-VEGF biotinylated detection antibody and streptavidin-HRP can be used for detection via the addition of a substrate solution and

colorimetric reading using a microtiter plate reader. The level of IL-8 or VEGF can be interpolated by non-linear regression analysis from a standard curve.

6.9.3. Migration and Invasion Assays

[0218] Migration and invasion assays are known to one of skill in the art. For example, migration assays can be designed to measure the chemotactic potential of the cell line, or its movement toward a concentration gradient of chemoattractants, such as, but not limited to, LPA or S1P. Invasion assays can be designed, for example, to evaluate the ability of the cell line to pass through a basement membrane, a key feature of metastasis formation.

[0219] Specific assays, known to one of skill in the art include a modified Boyden Chamber assay in which a cell suspension can be prepared in serum free medium and added to the top chamber. The concentration of cells to be added, for example, about 10^5 cells/ml is known to one of skill in the art. An appropriate dose of a chemoattractant can then be added to the bottom chamber. Following an incubation period, the number of cells invading the lower chamber can be quantified by methods known in the art. In one embodiment, Fluoroblok filter inserts can be used and the number of cells migrating to the lower chamber can be quantified by staining the filter inserts and detecting the fluorescence by any means known in the art. The level of fluorescence may be correlated with the number of migrating cells.

6.9.4. Proliferation Assay

[0220] Proliferation assays quantitate the extent of cellular proliferation in response to a stimulant, which, in the case of Edg-1 receptors, may be S1P. Cells can be plated and treated with the stimulant (*e.g.*, S1P) with or without any serum starvation. Stimulant doses may range from 0.1 to 10 μM and in any event may be readily determined by those of skill in the art. Typically, the cells can be treated for a period of a few hours to a few days before cellular proliferation is measured.

[0221] Specific methods to determine the extent of cell proliferation are known to one of skill in the art. For example, one method is bioluminescent measurement of ATP, which is present in all metabolically active cells. ATP can be extracted by addition of Nucleotide Releasing Reagent and its release can be monitored by the addition of the ATP Monitoring Reagent. An enzyme, such as

luciferase, which catalyzes the formation of light from ATP and luciferin, can be used to quantitate the amount of ATP present.

6.9.5. Cyclic AMP Assay

[0222] Because cAMP acts a second messenger in cell signaling, activating protein kinases that in turn phosphorylate enzymes and transcription factors, cAMP concentration is frequently indicative of the activation state of downstream signaling pathways. For GPCRs like the Edg receptors, coupling via a Gαi pathway results in inhibition of adenylyl cyclase activity, the key enzyme involved in breakdown of ATP and formation of cAMP. Thus, assays can be designed to measure inhibition of adenylyl cyclase activity, by first stimulating cAMP formation. One example of a compound, which stimulates cAMP formation is forskolin. Forskolin bypasses the receptor and directly activates adenylyl cyclase. Under these conditions, activation of a Gαi coupled receptor will inhibit forskolin-stimulated cAMP, and an antagonist at such a receptor will reverse the inhibition.

[0223] This assay can be performed by any means known to one of skill in the art. For example, cells can be plated and treated with or without any serum starvation. The cells may be initially treated with a compound, such as forskolin, to induce cAMP production. This is followed by the addition of an Edg-1 stimulator, for example, S1P. The dose of stimulator required is well known in the art, and could be in the range from 0.1-10 μM in serum free medium. Following an incubation period, the cells are lysed and the level of cAMP is determined.

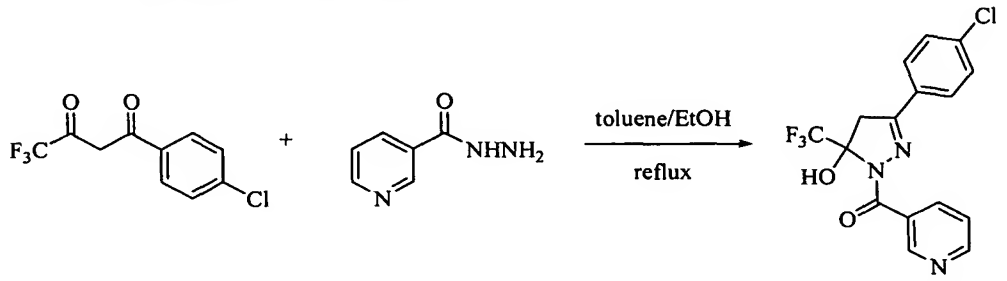
[0224] The cAMP assay can be performed by any means known to one of skill in the art, for example, by performing a competitive immunoassay. Cell lysates can be added to a plate precoated with anti-cAMP antibody, along with a cAMP-AP conjugate and a secondary anti-cAMP antibody. Detection can be performed by any appropriate means, including, but not limited to, using a substrate solution and chemiluminescent readout.

7. EXAMPLES

[0225] The invention is further defined by reference to the following examples, which describe in detail preparation of compounds and compositions of the invention and assays for using compounds and compositions of the invention. It will

be apparent to those skilled in the art that many modifications, both to materials and methods, may be practiced without departing from the scope of the invention.

7.1. Example 1: Synthesis of 101

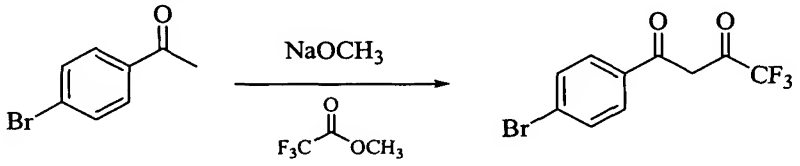


101

[0226] A solution of 1-(4-chlorophenyl)-4,4,4-trifluoro-1,3-butanedione (0.883 g, 3.17 mmol) and nicotinic acid hydrazide (0.448 g, 3.17 mmol) in ethanol (~2 mL) and toluene (50 mL) was heated at reflux. After 2 days the reaction mixture was reduced *in vacuo* and the crude residue was subjected to flash column chromatography over silica gel (98:2 CH_2Cl_2/MeOH). Recrystallization of the impure product from ethanol afforded 5-(4-chlorophenyl)-2-pyridin-3-ylmethyl-3-trifluoromethyl-3,4-dihydro-2*H*-pyrazol-3-ol (0.134 g, 11% yield) as a light yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.24 (m, 1H), 8.79 (m, 1H), 8.27 (m, 1H), 7.59 (m, 2H), 7.42 (m, 3H), 6.47 (s, 1H), 3.74 (d, 1H), 3.75 (d, 1H). ESI-MS *m/z*: 370 $[C_{16}H_{11}ClF_3N_3O_2 + H]^+$. m.p. 158 –160 °C.

7.2. Example 2: Synthesis of 102

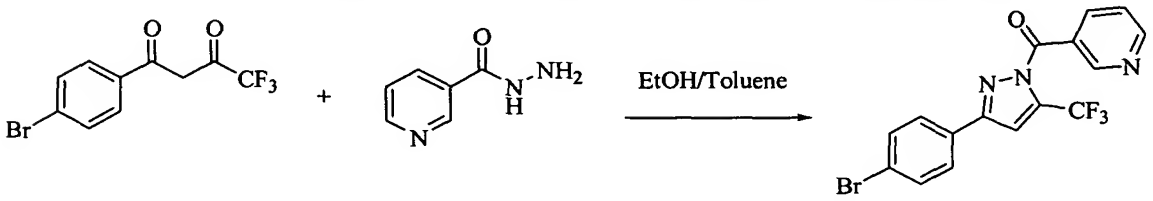
7.2.1. Preparation of 1-(4-Bromophenyl)-4,4,4-trifluoro-1,3-butanedione



[0227] To a solution of ethyl trifluoroacetate (81.0 mL, 0.452 mol) in methoxy *tert*-butyl ether (0.5 L) was slowly added sodium methoxide (30 wt % in methanol, 102 mL, 0.542 mol) at room temperature. The reaction exothermed and was cooled to room temperature with an ice bath. 4'-Bromoacetophenone (90.0 g, 0.452 mol) was added portion-wise to the reaction mixture over 10 min. and the reaction mixture was stirred overnight at room temperature. The organic layer was washed with

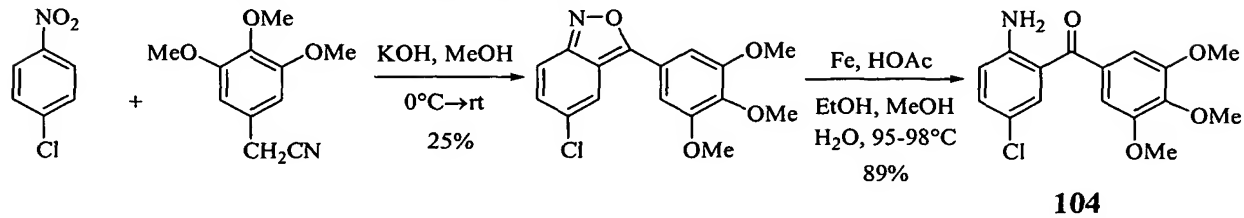
hydrochloric acid (3N, 300 mL), brine (100 mL), dried (magnesium sulfate), filtered, and concentrated *in vacuo*. The residue was recrystallized from hexanes five times to provide 1-(4-bromophenyl)-4,4,4-trifluoro-1,3-butanedione (96.5 g, 72%) as a pale yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.9 (s, 1H), 7.83–7.75 (m, 2H), 7.68–7.60 (m, 2H), 6.54 (s, 1H).

7.2.2. Preparation of [3-(4-Bromophenyl)-5-trifluoromethylpyrazol-1-yl]pyridin-3-yl-methanone



[0228] A mixture of 1-(4-bromophenyl)-4,4,4-trifluoro-1,3-butanedione (55.2 g, 0.187 mol) and nicotinic acid hydrazide (25.6 g, 0.187 mol) in absolute ethanol (0.7 L) was stirred at reflux overnight. The reaction mixture was allowed to cool to room temperature and then was concentrated *in vacuo*. The crude residue was taken up in toluene (0.7 L) and was stirred at reflux for two days. The mixture was allowed to cool and then was concentrated *in vacuo*. The residue was treated with methoxy *tert*-butyl ether (0.4 L) and stirred at 55 °C for thirty min. The solids were filtered off and the mother liquor concentrated *in vacuo*. The crude residue was purified by chromatography (silica gel, 0.5% methanol/methylene chloride) to obtain [3-(4-bromophenyl)-5-trifluoromethylpyrazol-1-yl]pyridin-3-yl-methanone (13.0 g, 17%) as a white solid: mp 150-152 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 9.11–8.92 (m, 1H), 8.69–8.58 (m, 1H), 8.38–8.20 (m, 1H), 7.74–7.42 (m, 5H), 3.95 (d, 1H), 3.60 (d, 1H); ESI MS *m/z* 414 $[C_{16}H_{11}BrF_3N_3O_2 + H]^+$.

7.3. Example 3: Synthesis of 104



104

[0229] Potassium hydroxide pellets (85%, 5.76g, 87.3 mmol, 17.5 equiv) were dissolved in methanol (12 mL). The mixture was cooled with an ice bath, and a solution of *p*-chloronitrobenzene (788 mg, 5 mmol) and

3,4,5-trimethoxyphenylacetonitrile (Acros, 1.140 g, 5.5 mmol) in methanol (15 mL) was added dropwise (ref. Davis, R.B.; Pizzini, L.C. *J. Org. Chem.* **1960**, *25*, 1884-1888). The dark purple reaction mixture was slowly warmed up to room temperature overnight and then poured into water (250 mL). The precipitates were collected by a suction filtration, washed several times with water, air-dried, and subject to flash column chromatography over silica gel using ethyl acetate/hexanes (1:10, 1:6, and 1:4). 5-Chloro-3-(3,4,5-trimethoxy-phenyl)benzo[*c*]isoxazole was isolated as a slightly yellow solid (405 mg, 25% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (m, 1H), 7.59 (m, 1H), 7.27 (m, 1H), 7.16 (s, 2H), 4.01 (s, 6H), 3.96 (s, 3H). APCI-MS: *m/z* 320 $[C_{16}H_{14}ClNO_4 + H]^+$. m.p. 119 – 120°C.

[0230] A mixture of iron powder (183 mg, 3.28 mmol, 3 equiv), ethanol (4 mL), water (2 mL), and acetic acid (6 mL) was preheated to 60°C (oil bath). 5-Chloro-3-(3,4,5-trimethoxyphenyl)-benzo[*c*]isoxazole (350 mg, 1.10 mmol) in methanol (10 mL) was then added and the reaction mixture was stirred for 30 min at that temperature and then overnight at 95-98°C. Most of the solvents were removed by rotary evaporation and the residue was diluted with dichloromethane (100 mL). Insoluble inorganics were removed by filtration and the filtrate solution was concentrated by rotary evaporation. The resulting solid was subject to flash column chromatography over silica gel using ethyl acetate/hexanes (1:10, 1:6, 1:4, and 1:2). (2-Amino-5-chlorophenyl)-(3,4,5-trimethoxyphenyl)methanone was isolated as a yellow solid (315 mg, 89% yield). ^{1}H NMR (500 MHz, CD_3OD) δ 7.35 (m, 1H), 7.24 (m, 1H), 6.91 (s, 2H), 6.83 (m, 1H), 3.86 (s, 6H), 3.85 (s, 3H). APCI-MS: *m/z* 322 $[C_{16}H_{16}ClNO_4 + H]^+$. m.p. 141 – 142°C.

7.4. Example 4: Selective Inhibition of the Edg-1 Receptor by Compounds 101, 102, and 103

[0231] **101, 102** and **103** are representatives of a series of compounds that demonstrate inhibition of Edg-1 stimulated S1P responses. The compounds were tested in HTC cells expressing human Edg-1 receptors. The rat hepatoma cell line, HTC, does not express any detectable levels of any of the known Edg receptors. Therefore, HTC proved to be a useful system because Edg-1 could be tested in isolation when recombinantly introduced into these cells. The compounds were tested in this recombinant system first, and subsequently tested in cell lines expressing Edg-1 (in addition to other Edg receptors).

[0232] Figure 1 demonstrates that **101** specifically inhibited Edg-1 receptors. **101** did not inhibit LPA-stimulated calcium increases in HTC cells expressing Edg-2, Edg-4 or Edg-7 receptors and also did not inhibit S1P-stimulated calcium increases in HTC cells expressing Edg-3, Edg-5, Edg –6 or Edg-8 in concentrations as high as 20 μM.

[0233] Figure 2 demonstrates that another Edg-1 antagonist, **102** specifically inhibited Edg-1 receptors. **102** did not inhibit LPA-stimulated calcium increases in HTC cells expressing Edg-2, Edg-4 or Edg-7 receptors and also did not inhibit S1P-stimulated calcium increases in HTC cells expressing Edg-3, Edg-5, Edg –6 or Edg-8 in concentrations as high as 20 μM.

[0234] Figure 3 demonstrates that another Edg-1 antagonist, **103** specifically inhibited Edg-1 receptors. **103** did not inhibit LPA-stimulated calcium increases in HTC cells expressing Edg-2, Edg-4 or Edg-7 receptors and also did not inhibit S1P-stimulated calcium increases in HTC cells expressing Edg-3, Edg-5, Edg –6 or Edg-8 in concentrations as high as 20 μM.

[0235] Figure 4 demonstrates that the Edg-1 antagonist **101** inhibits S1P induced calcium responses in a dose-dependent manner in human umbilical vein endothelial cells. The calcium mobilization assay was performed according to Section 7.10, below.

[0236] Figure 5 demonstrates that the Edg-1 antagonist **102** inhibits S1P induced calcium responses in a dose-dependent manner in human umbilical vein endothelial cells. The calcium mobilization assay was performed according to Section 7.10, below.

[0237] Selectivity of **101, 102** and **103** for Edg-1 is also demonstrated in Table 2. Table 2 demonstrates the selectivity of **101, 102,** and **103** for Edg-1 relative to other Edg receptors. Table 3 is a list of targets, including GPCRs and ion channels, against which **101** (10 μM) showed no activity in standard binding assays. The radioligand binding assays were conducted as described in Section 7.15.

7.5. Example 5: 101 and 301 Inhibit S1P Stimulated Chemotaxis by HUVECs

[0238] As shown above, **101** selectively inhibits Edg-1 mediated S1P responses. In addition to Edg-1, other Edg receptors are also implicated in S1P stimulated chemotaxis, including Edg-3 and Edg-6. To assess the relative roles of the

Edg-1 receptors and Edg-3 receptors in S1P stimulated chemotaxis, the ability of S1P to evoke trans-matrigel chemotaxis was tested in the presence of the Edg-1 antagonist **101**, the Edg-3 antagonist **301**, and the combination of **101** and **301**. These experiments were performed according to the migration and invasion assay protocols described in Section 7.12, below. The Edg-3 antagonist **301**, 3-Methyl-2-phenyl-quinoline-4-carboxylic acid 4-fluoro-benzylamide, is extensively described in co-pending U.S. Application No. 10/390,426, which is incorporated by reference in its entirety. **301** is identified as compound 101 in U.S. Application No. 10/390,426. Further, **301** is commercially available from Specs (The Netherlands) as compound number AK-968/12971392.

[0239] Figure 10 presents the results of these experiments, showing that both compound **101** and compound **301** inhibit S1P-stimulated chemotaxis. In addition, **101** is a more potent inhibitor of S1P-stimulated chemotaxis than **301**. Further, the results indicate that the inhibitory effect of **101** and **301** are additive, suggesting that combinations of Edg-1 agonists and/or antagonists and agonists and/or antagonists of Edg-3 or other Edg receptors may be useful in the methods of the invention.

7.6. Example 6: 104 is a Selective Agonist of the Edg-1 Receptor

[0240] Selectivity of the Edg-1 agonist **104** is shown in Table 4. Table 4 demonstrates the selectivity of **104** for Edg-1 relative to other Edg receptors. **104** did not elicit a calcium response in HTC cells expressing Edg-2, Edg-3, Edg –4, Edg-5, Edg –6, Edg-7 or Edg-8 receptors in concentrations as high as 25 μM. The calcium mobilization assay was performed according to Section 7.10, below.

7.7. Example 7: S1P and Edg-1 Agonists Regulate Chemotactic Response to Exodus-2

[0241] Edg-1 receptors and Edg-6 receptors are strongly expressed in naïve CD4 and CD8 cells, but much less strongly expressed in CD4 and CD8 cells that have been exposed to antigen. Without intending to be bound to any theory or mechanism of action, it is believed that the Edg-1 and Edg-6 receptors regulate the sensitivity of CD4 and CD8 lymphocytes cytokines that bring the lymphocytes to compartments where they are exposed to antigen. As part of this mechanism, Edg-1 modulates lymphocytes' chemotactic response to cytokines, including, for example, exodus-2.

[0242] To assess this modulation and to test the effects of Edg-1 agonists and antagonists on this effect, murine CD4 T cells chemotactic responses to exodus-2

were tested in the presence of S1P and the Edg-1 agonist **104.** These experiments were performed according to the migration and invasion assay protocols described in Section 7.12, below.

[0243] Figure 11 presents the results of these experiments. It shows that both S1P and **104** exhibit a bell-shaped dose dependant response curve to chemotaxis of murine CD4 T cells to exodus-2. At low and high concentrations of S1P and **104**, the chemotactic response to exodus-2 is desensitized, but moderate concentrations of S1P and **104** stimulate the chemotactic response to exodus-2. Further, S1P and **104** exhibit the same profile on the chemotactic response to exodus-2, but higher concentrations of **104** than S1P are required to exert the same effect on the chemotactic response.

[0244] The effects of Edg-1 antagonists on S1P regulation of the chemotactic response to exodus-2 were also assessed. This experiment was performed according to the migration and invasion assay protocols described in Section 7.12, below. Figure 12 shows the results of experiments testing the ability of the Edg-1 antagonists FTY720 and **102** to reverse S1P-mediated stimulation and desensitization of the chemotactic response to exodus-2. At moderate concentrations of S1P, FTY720 and **102** inhibit S1P-mediated stimulation of the chemotactic response to exodus-2. At high concentrations of S1P, FTY720 and **102** reverse S1P-mediated desensitization of the chemotactic response to exodus-2. Thus, these Edg-1 antagonists can antagonize both aspects of the regulation by S1P of exodus-2-mediated chemotaxis.

7.8. Example 8: Inhibition or Stimulation of S1P-Mediated Migration by 101, 102 and 104

[0245] S1P is a strong chemoattractant to human PBMCs. This effect is believed to be mediated by S1P receptors, notably Edg-1 (Mandala *et al.*, Science 2002; Lynch *et al.*, JBC 2002; Goetzl, personal communication). Several migration assays were performed to test this hypothesis and to determine the effect of Edg-1 agonists and antagonists on S1P mediated chemotaxis. The migration assays were performed according to the protocol described in Section 7.12, below. The results of theses assays are presented in Figure 6, Figure 7, Figure 8, Figure 9 and Figure 13.

[0246] Figure 13 shows that S1P attracts human PMBCs in a dose dependent manner. Figure 7 demonstrates that the Edg-1 agonist **104** stimulates invasion of Human Umbilical Vein Endothelial cells at concentrations between 0.5-100 μM,

though this stimulation is significantly less potent that S1P. In addition, Figure 9 shows that **104** also stimulates migration of human peripheral blood mononuclear cells at micromolar concentrations. Thus, **104** can attract cells expressing the Edg-1 receptor in a manner similar to S1P.

[0247] Further, Figures 6 and 8 show that Edg-1 antagonists can inhibit S1P and **104**-mediated chemotaxis, indicating that the S1P chemoattractant effect is likely mediated by the Edg-1 receptor. Figure 6 shows that the Edg-1 antagonist **102** inhibits S1P-induced matrigel invasion, while Figure 8 demonstrates that the Edg-1 antagonist **101** inhibits **104**-induced matrigel invasion.

7.9. Example 9: The Edg-1 Receptor Regulates Lymphocyte Trafficking *in Vitro* and *in Vivo*

[0248] To further elucidate the role of the Edg-1 receptor in lymphocyte trafficking, migration of mouse T cells was analyzed in both *in vitro* and *in vivo* models. Migration of mouse T cells was first tested in Transwell chambers (Costar, Cambridge, MA) with polycarbonate fibers having a pore width of 8 μm, separated by a layer of matrigel. Stimulants S1P or exodus-2 were separately added to the bottom chamber. The effects of exodus-2 were tested alone, with S1P, and with S1P and Edg-1 antagonists **101** and **102**.

[0249] Figure 14 presents the results of this experiment. The data show that high concentrations of S1P alone do not stimulate T cell migration, while exodus-2 does stimulate T cell migration. However, **101** and **102** enhance exodus-2-stimulated migration even in the presence of S1P. Thus, Edg-1 antagonists can reverse S1P-mediated inhibition of migration of T-cells to exodus-2 *in vitro*.

[0250] To test migration of mouse T cells *in vivo*, the ability of exodus-2 to attract mouse lymphocytes was assessed in the mouse dorsal air-pouch model. In these experiments, 0.5 ml 1μM exodus-2 was introduced into a dorsal air-pouch produced by injecting 5 ml of filtered air subcutaneously. Mouse splenic CD4 T cells were fluorescently labeled with FM-DiI (Molecular Probes, Inc., Eugene, OR), then treated with S1P (1 μM), **102**, or **104**. The treated CD4 T cells were introduced into the peritoneal cavity. After 24 hours, lavage (1 ml) from the dorsal air pouch was assayed for fluorescence.

[0251] Figure 15 presents the results of this experiment. The data indicate that the Edg-1 receptor, and compounds that modulate the Edg-1 receptor, can

regulate lymphocyte tracking in the mouse air-pouch model. In particular, PBS-treated lymphocytes migrate to the chemoattractant exodus-2, while lymphocytes treated with S1P or the Edg-1 agonist **104** do not. Thus, the *in vivo* mouse dorsal air-pouch animal model confirms that S1P or **104** stimulation of the Edg-1 receptor desensitizes the lymphocytes to attraction by exodus-2. Further, this S1P-mediated desensitization can be reversed by co-treatment with the selective Edg-1 antagonist **102**.

[0252] These experiments confirm that S1P regulates the ability of lymphocytes to respond to various chemotactic factors, as exemplified by exodus-2. These experiments further indicate that S1P-dependent regulation of the ability to respond to such chemotactic factors is mediated at least in part by the Edg-1 receptor. Finally, the experiments show that use of selective Edg-1 agonists and antagonists can successfully manipulate this regulation, permitting increased or decreased lymphocyte homing and trafficking as desired.

7.10. Example 10: Intracellular Calcium Measurement Assays

[0253] LPA receptors such as Edg-1, couple to calcium effector pathways, and result in increases in intracellular calcium following receptor activation (An, 1998, J. Cell Biochem. Supp 30-31:147-157). This biological response lends itself to a very efficient, high-throughput screen using a Fluorescence Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA). The FLIPR system is a real-time, cell-based assay system with continuous fluorescence detection using a cooled CCD camera. The FLIPR system was used to develop an Edg-1 receptor screen. Rat hepatoma cells stably expressing Edg-1/3i3 chimera or Edg-1 and Gqi were plated on 384-well plates and loaded with a calcium dye loading kit (Molecular Devices, Sunnyvale, CA) for 1 hour at room temperature. Cells were then placed on the FLIPR^{384} (Molecular Devices, Sunnyvale, CA) and excited by an argon laser at 488 nm. The data for the entire 384-well plate was updated every second. An integrated robotic pipettor allowed for simultaneous compound addition into each individual well in the plate.

7.11. Example 11: IL-8 and VEGF Assays

[0254] IL-8 and VEGF assays were performed by standard enzyme-linked immunosorbent assay ("ELISA") techniques. Cells were cultured in a 96 well format, serum starved overnight, and treated with LPA or S1P (doses range from 0.1-10 μM

in serum free medium) for 24 hours. Cell supernatants were then collected to measure the amount of IL-8 secreted.

[0255] The assay was a standard sandwich ELISA in which an anti-IL-8 or VEGF capture antibody was adsorbed to a plastic dish. Cell supernatants containing IL-8 or VEGF were added to the dish, and then an anti-IL-8/VEGF biotinylated detection antibody and streptavidin-HRP were added.

[0256] Detection was via the addition of a substrate solution and colorimetric reading using a microtiter plate reader. The level of IL-8 or VEGF was interpolated by non-linear regression analysis from a standard curve.

[0257] All reagents were from R&D Systems, Minneapolis, MN: MAB208 and AF-293-NA (capture antibody for IL-8 and VEGF respectively), BAF208 and BAF-293 (detection Ab for IL-8 and VEGF respectively), 208-IL-010 and 293-VE-010, (recombinant human IL-8 protein standard and recombinant human VEGF protein standard respectively), DY998 (streptavidin-HRP), DY999 (substrate solution).

7.12. Example 12: Migration and Invasion Assays

[0258] Cells were plated in a 24 well format using Fluoroblok filter insert plates (8 μM pore size) or Fluoroblok matrigel coated filter insert plates (Becton Dickinson, San Diego, CA). The assay was a modified Boyden Chamber assay in which a cell suspension (1 x 10^5 cells/ml) was prepared in serum free medium and added to the top chamber. LPA or S1P (doses ranged from 0.1-10 μM in serum free medium) was added to the bottom chamber. Following a 20-24 hour incubation period, the number of cells migrating or invading into the lower chamber was quantitated by transferring the filter insert into a fresh 24-well plate containing 4 μg/ml calcein AM (Molecular Probes, Sunnyvale, CA) in Hank's Balanced Salt Solution and staining for one hour.

[0259] Detection was via fluorescent readout at 450 nm excitation/530 nm emission using a fluorimeter. The level of fluorescence correlated with cell number.

[0260] For most cells types, no further manipulation was required. For CaOV3 human ovarian cancer cells, however, it was necessary that the cells be serum starved overnight prior to preparing the cell suspension. In addition, the filter inserts were coated with a solution of 1 mg/ml rat-tail Collagen I (BD, San Diego, CA).

[0261] Human PBMCs are isolated from the buffy coats of healthy donors from the Stanford Blood Center. The blood is washed one time with PBS, then layered over a Ficoll gradient and centrifuged for 45 minutes at 400 g. The white mononuclear cell layer is removed and washed three times with PBS. The cells are then resuspended in complete RPMI containing 5 μg/ml PHA and 100 ng/ml recombinant human IL-2 and cultured in T175 flasks at a cell density should of 1 X 10^6 cells/ml. For maintenance in culture, cells are washed and resuspended in fresh RPMI + IL-2 every 3-5 days.

7.13. Example 13: Proliferation Assay

[0262] Cells were plated in a 96 well format. Treatments were performed directly without any serum starvation, and typically included LPA or S1P doses in a range from 0.1-10 μM in serum free medium. Cells were treated for 24-48 before the extent of cellular proliferation was measured.

[0263] The assay was performed using the ViaLight HS kit from BioWhittaker, Rockland, ME, which is based upon the bioluminescent measurement of ATP that is present in all metabolically active cells. The reaction utilized an enzyme, luciferase, which catalyzes the formation of light from ATP and luciferin. The emitted light intensity was linearly related to the ATP concentration, which correlated with cell number.

[0264] Measurement of cell proliferation required the extraction of ATP by the addition of Nucleotide Releasing Reagent, followed by the addition of the ATP Monitoring Reagent (both provided in kit). Detection was via chemiluminescence using the EG&G Berthold Luminometer, Gaithersburg, MD.

7.14. Example 14: cAMP Assay

[0265] Cells were plated in a 96 well format. Treatments were performed directly without any serum starvation. The cells were treated with forskolin to induce cAMP production, followed by LPA or S1P doses in the range from 0.1-10 μM in serum free medium. Following a 30-minute incubation period, the cells were lysed and the level of cAMP was determined.

[0266] The cAMP assay was performed using the Tropix cAMP-Screen (Applied BioSystems, Foster City, CA). The screen is a competitive immunoassay that utilizes a 96 well assay plate precoated with an anti-cAMP antibody. Cell lysates

were added to the precoated plate, along with a cAMP-AP conjugate and a secondary anti-cAMP antibody.

[0267] Detection was performed using a substrate solution and chemiluminescent readout. The level of chemiluminescence was inversely proportional to the level of cAMP and was calculated from a standard curve.

7.15. Example 15: Pharmacology Profiling (Selectivity Assays)

[0268] In order to test the selectivity of compounds, various enzyme assays as well as radioligand binding assays were performed using numerous non-Edg receptor targets as listed below.

[0269] A radioligand binding assay was performed using adrenergic α_1 according to the method of Greengrass and Bremner **1979**, *Eur. J. Pharmacol.* 55:323-326. A radioligand binding assay was performed using adrenergic α_2 according to the method of Boyajian and Leslie, **1987**, *J. Pharmacol. Exp. Ther.* 241:1092-1098. A radioligand binding assay was performed using adrenergic β according to the method of Feve *et al.*, **1994**, *Proc. Natl. Acad. Sci. USA* 91:5677-5681. A radioligand binding assay was performed using angiotensin AT2 according to the method of Whitebread *et al.*, **1991**, *Biochem. Biophys. Res. Comm.* 181:1365-1371. A radioligand binding assay was performed using calcium channel Type L, dihydropyridine according to the method of Ehlert *et al.*, **1982**, *Life Sci.* 30:2191-2202. A radioligand binding assay was performed using dopamine D_{2L} according to the method of Bunzo *et al.*, **1988**, *Nature* 336:783-787. A radioligand binding assay was performed using endothelin ET_A according to the method of Mihara *et al.*, **1994**, *J. Pharmacol. Exp Ther.* 268:1122-1127. A radioligand binding assay was performed using histamine H_1 Central according to the method of Hill *et al.*, **1978**, *J. Neurochem.* 31:997-1004. A radioligand binding assay was performed using Muscarinic non-selective, Central according to the method of Luthin and Wolfe, **1984**, *J. Pharmacol. Exp. Ther.* 228:648-655. A radioligand binding assay was performed using serotonin 5-HT1, non-selective according to the method of Middlemiss, **1984**, *Eur. J. Pharmacol.* 101:289-293).

7.15.1. Radioligand Binding assays

1. **Adrenergic α_1, non-selective** (Broadhurst *et al.*, **1988**, *Life Sci.* 43:83-92). Source: Wistar Rat brain

Ligand: 0.25 nM ^{3}H Prazosin

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 30 minutes at 25 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, 0.1% ascorbic acid, 10 μM

NonSpecific Ligand: 0.1 μM Phentolamine

K_d: 0.29 nM *

B_{max}: 0.095 pmol/mg Protein*

Specific Binding: 90% *

Quantitation Method: Radioligand Binding

Significance Criteria: ≥ 50% of max stimulation or inhibition

2. Adrenergic α_2 (Boyajian and Leslie, **1987**, *J. Pharmacol. Exp. Ther.* 241:1092-1098).

Source: Wistar rat cerebral cortex

Ligand: 0.7 nM ^{3}H Rauwolscine

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 30 minutes at 25 ^{0}C

Incubation Buffer: 20 mM HEPES, 2.5 mM Tris-HCl, pH 7.4 at 25 ^{0}C

NonSpecific Ligand: 1 μM Yohimbine

K_d: 7.8 nM *

B_{max}: 0.36 pmol/mg Protein*

Specific Binding: 80% *

Quantitation Method: Radioligand Binding

Significance Criteria: ≥ 50% of max stimulation or inhibition

3. Adrenergic β (Feve *et al.*, **1994**, *Proc. Natl. Acad. Sci. USA* 91:5677-5681).

Source: Wistar rat brain

Ligand: 0.25 nM ^{3}H Dihydroaplenolol

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 20 minutes at 25 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, pH 7.4

NonSpecific Ligand: 1μM S(-)-Propranolol

K_d: 0.5 nM *

B_{max}: 0.083 pmol/mg Protein*

Specific Binding: 85% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

4. Angiotensin AT2 (Whitebread *et al.*, **1991**, *Biochem. Biophys. Res. Comm.* 181:1365-1371).

Source: Human recombinant Hela cells

Ligand: 0.025 nM ^{125}I CGP-42112A

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 3 hours at 37 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1% BSA, 1 mM EDTA, pH 7.4

NonSpecific Ligand: 10 μM [Sar^1, Ile^8]-Ang II

K_d: 0.012 nM *

B_{max}: 2.9 pmol/mg Protein*

Specific Binding: 90% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

5. Calcium Channel Type L, Dihydropyridine (Ehlert *et al.*, **1982**, *Life Sci.* 30:2191-2202).

Source: Wistar Rat cerebral cortex

Ligand: 0.1 nM ^{3}H Nitrendipine

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 90 minutes at 25 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, pH 7.7

NonSpecific Ligand: 1 μM Nitrendipine

K_d: 0.18 nM *

B_{max}: 0.23 pmol/mg Protein*

Specific Binding: 91% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

6. Dopamine D_{2L} (Bunzo *et al.*, **1988**, *Nature* 336:783-787).

Source: Human recombinant CHO cells

Ligand: 0.16 nM ^{3}H Spiperone

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 2 hours at 25 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.4 mM ascorbic acid, 0.001% BSA

NonSpecific Ligand: 10 μM Haloperidol

K_d: 0.08 nM *

B_{max}: 0.48 pmol/mg Protein*

Specific Binding: 85% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

7. Endothelin ET_A (Mihara *et al.*, **1994**, *J. Pharmacol. Exp Ther.* 268:1122-1127).

Source: Human recombinant CHO cells

Ligand: 0.03 nM ^{125}I Endothelin

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 2 hours at 37 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, pH 7.4, 0.5 mM CaCl2, 0.1% bacitracin, 0.05% Tween-20, 1 mg/ml BSA

NonSpecific Ligand: 0.1 μM Endothelin-1

K_d: 0.048 nM *

B_{max}: 0.35 pmol/mg Protein*

Specific Binding: 90% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

8. Histamine H_1, Central (Hill *et al.*, **1978**, *J. Neurochem.* 31:997-1004).

Source: Guinea pig cerebellum

Ligand: 1.75 nM ^{3}H Pyrilamine

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 60 minutes at 25 ^{0}C

Incubation Buffer: 50 mM K-Na phosphate buffer pH 7.4 at 25^0C

NonSpecific Ligand: 1 μM Pyrilamine

K_d: 0.23 nM *

B_{max}: 0.198 pmol/mg Protein*

Specific Binding: 90% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

9. Muscarinic non-selective, Central (Luthin and Wolfe, **1984**, *J. Pharmacol. Exp. Ther.* 228:648-655).

Source: Wistar rat cerebral cortex

Ligand: 0.29 nM ^{3}H Quinuclidinyl benzilate

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 60 minutes at 25 ^{0}C

Incubation Buffer: 50 mM Na-K Phosphate, pH 7.4

NonSpecific Ligand: 0.1 μM Atropine

K_d: 0.068 nM *

B_{max}: 1.4 pmol/mg Protein*

Specific Binding: 97% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

10. Serotonin 5-HT1, non-selective (Middlemiss, **1984**, *Eur. J. Pharmacol.* 101:289-293).

Source: Wistar rat cerebral cortex

Ligand: 2 nM ^{3}H Serotonin (5-HT) Trifluoroacetate

Vehicle: 0.4 % DMSO

Incubation Time/Temp: 10 minutes at 25 ^{0}C

Incubation Buffer: 50 mM Tris-HCl, 0.1% ascorbic acid, 10 μM pargyline, 4 mM CaCl2, pH 7.6

NonSpecific Ligand: 10 μM 5-HT (Serotonin)

K_d: 0.61 nM *

B_{max}: 0.58 pmol/mg Protein*

Specific Binding: 80% *

Quantitation Method: Radioligand Binding

Significance Criteria: $\geq$ 50% of max stimulation or inhibition

* Historical Values

[0270] Finally, it should be noted that there are alternative ways of implementing both the present invention. Accordingly, the present embodiments are to be considered as illustrative and not restrictive, and the invention is not to be limited to the details given herein, but may be modified within the scope and equivalents of the appended claims.

[0271] All publications and patents cited herein incorporated by reference in their entirety.